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(21) International Application Number: <b>PCT/US96/07666</b> (22) International Filing Date: <b>24 May 1996 (24.05.96)</b> (30) Priority Data: <b>113926 31 May 1995 (31.05.95) IL</b> (71) Applicant (for all designated States except US): <b>RAMOT UNIVERSITY AUTHORITY FOR APPLIED RESEARCH AND INDUSTRIAL DEVELOPMENT LTD. [IL/IL]; 32 Haim Levanon Street, 60075 Ramat-Aviv (IL).</b> (71) Applicant (for MW only): <b>SHOSHAN, Herbert, Z. [US/IL]; 344 Agur, Maccabim (IL).</b> (72) Inventors; and (75) Inventors/Applicants (for US only): <b>KORENSTEIN, Rafi [IL/IL]; 59 Sireni Street, 76240 Rehovot (IL). DASCALU, Avi [IL/IL]; 20 Mosenzon Street, 62965 Tel-Aviv (IL).</b> (74) Agent: <b>GERSTENZANG, William, C.; Sprung Horn Kramer &amp; Woods, 660 White Plains Road, Tarrytown, NY 10591 (US).</b>		(81) Designated States: <b>AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).</b>  <b>Published</b> <i>With international search report.</i>	

(54) Title: **CONTRAST MEDIUM AND ITS USE**

(57) Abstract

Contrast medium comprising an effective amount of a contrast agent and a protecting agent wherein the protecting agent is active in protecting against possible damaging effects of the contrast medium.

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## CONTRAST MEDIUM AND ITS USE

## FIELD OF THE INVENTION

The present invention is generally in the field of contrast media. More specifically, the present invention relates to a contrast medium with improved patient's tolerance.

5

## PRIOR ART

The following is a list of references which are believed to be pertinent as prior art to the present invention:

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2. Sovak M., Handbook of Experimental Pharmacology, 73, 1-19, Springer-Verlag, Berlin (1984).
- 15 3. Gmelin E., et al., Recent developments in nonionic contrast media, pp. 67-69, Thieme Med. Pub. Inc., N.Y. (1989).
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- 20 5. Hou S.H., et al., Am. J. Med., 74, 243-248, (1983).
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- 5 9. Cala, M.C., et al., Comp. Biochem. Physiol., 90A, 551-555, (1988).
- 10 10. Busa W.B., Annu. Rev. Cell Physiol., 48, 389-402, (1986).
11. Dascalu A., Peer A., Academic Rad., 1, 145-150, (1994).

The above references will be referred to herein by indicating, within brackets, the number from the above list.

15 **BACKGROUND OF THE INVENTION**

Contrast Media (hereinafter "CM") are routinely used in various imaging procedures. Such procedures include visualization of blood vessels in cardiac angiography, either by x-ray imaging or by Magnetic Resonance Imaging (MRI), intravenous urography (kidney imaging), computerized tomography and neurologic visualization of the spinal cord, the brain, etc. In the U.S.A. alone, there are more than 10 million x-ray radiologic examinations using CM, performed each year. 5 to 10% of these procedures are accompanied by clinical side effects; in 1 out of 1000-2000 of such procedures, there occurs a life threatening complication.

25 The currently used CM in the x-ray imaging procedure can be grouped, on the basis of their osmolarity, to such which have a low osmolarity (hereinafter "LOCN") and such having a high osmolarity (hereinafter "HOCM"). It should be noted that both LOCM and HOCM have an osmolarity which is above that of the blood. HOCM have a typical osmolarity of about 1500 - 2000 mOsm/kg and LOCM have an osmolarity within the range of 300 - 700 mOsm/kg. Adverse side effects associated with CMs include such which result from the high osmolarity. The introduction of LOCMs, which are the new generation of CMs, was meant to counter some of these side effects. It should be noted that one big disadvantage of LOCMs

is their high price-tag (about 5-10 times that of HO�M). Therefore, there is a dispute of the kinds of x-ray CMs which should be used<sup>(1)</sup>.

The visualization which is the outcome of CM injection into the blood, results from a local dispersion of the high iodine atom concentration contained in the CM, from the high osmolarity, as well as from an increased viscosity in the blood vessels of the visualized organ<sup>(2)</sup>. In procedures wherein the CMs are introduced into the blood, this is achieved either by injection into the blood vessels or by catheterization.

The MRI method for visualization of blood vessels comprises the injection of a paramagnetic substance dissolved in a hyperosmotic CM to the region to be visualized.

Individuals subjected to procedures involving the use of CMs, are exposed to several hazards, depending on the CM used, including:

1. **Hyperosmotic damage:** Typically 100-200 ml of CM are injected into a total plasma volume of 5 liters within a period of several minutes. Cells such as endothelial cells, red and white blood cells, cells within the kidney, etc., are exposed to a hyperosmotic solution, reaching 200-2000 mOsm/kg at the site of injection, as compared to the osmolarity of the blood with its 300 mOsm/kg, giving rise to a hyperosmotic shock which may elicit related damages. In the following description the term "hyperosmotic CM" will refer to any CM having osmolarity higher than the blood osmolarity which is typically 300 mOsm/kg.

2. **Iodine specific toxicity:** In an x-ray visualization procedure typically 30-40 grams of iodine (included within the contrast media) are injected into the blood within the period of 2-10 minutes. It should be noted that target visualization requires a minimum accumulation of 15-20 mg of iodine/ml in the target tissue<sup>(3)</sup> and this is the reason that the initial iodine concentration in the CM is relatively high in the range of 300-420 mg iodine/ml.

3. **Kidney damage:** The iodine load to which the kidney is exposed and which it has to secrete is a potential cause for renal damage<sup>(4)</sup>. It is generally believed today that 12% of all patients which are injected with an x-ray CM, encounter renal complications<sup>(5)</sup>. A recent study shows that during cardiac

catheterization procedure, 9% of low risk and 16% of high risk patients develop renal failure<sup>(6)</sup>. Various studies made with CMs have shown that exposure of cells to x-ray CM causes the cell damages<sup>(7,8,9,10)</sup>.

## 5 SUMMARY OF THE INVENTION

It is an object of the present invention to provide a novel contrast medium composition. It is particularly an object of the invention to provide a contrast medium composition with lower hazardous side effects as compared to hitherto used CM compositions.

10 It is more particularly the object of the present invention to provide such a composition comprising, in addition to the contrast medium, also other agents which protect tissue against damages which can be inflicted by the CM by either direct cytotoxic effects or hyperosmotic effects. According to a particular embodiment, it is the object of the present invention to provide  
15 such a composition containing endothelial and kidney cells protecting agents.

It is further the object of the present invention to provide a method for protecting tissue and organs within the body from harmful effect which may otherwise be inflicted by the CM.

In the following description, the term "contrast agent" or "CA" will  
20 be used to denote an agent which absorbs or reacts in another way with the electromagnetic irradiation used in a body visualization procedure. The CA, which is injected into the circulation, may be an agent, such as an iodine containing substance, which blocks x-ray irradiation and can thus be used for the visualization of blood vessels or other body fluid-containing organs or  
25 tissue in x-ray visualization procedure. The CA may also be a paramagnetic substance used in an MRI visualization procedure.

The term "contrast medium" or "CM" will be used to denote a liquid composition comprising a CA which is injected into the circularization prior to visualization process. The CM is typically a hyperosmotic composition.  
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The term "protecting agent" or "PA" will be used to denote an agent which in accordance with the present invention is incorporated into a

CM, for the purpose of protecting tissue or organs, fully or partially, against some or all of the hazardous effects of CM's noted above. The CM in accordance with the present invention thus comprises both a CA and a PA.

In the following the term "effective amount" will be used to denote an amount of an agent sufficient to achieve the desired effect. In the case of a CA, the effective amount is an amount sufficient to achieve the desired contrast. In the case of a PA, an effective amount is an amount sufficient to achieve a protecting effect.

The present invention provides by a first of its aspects, a contrast medium, comprising an effective amount of a contrast agent, and an effective amount of a protecting agent being an agent active in protecting against possible damaging effects of the contrast medium, said protecting agent being one or more members selected from the group consisting of (i) non-steroidal anti-inflammatory drugs (NSAID), (ii) agents which induce cells to generate nitrous oxides (NO), (iii) polysaccharides capable of sealing the intercellular spaces which exist between endothelial cells, (iv) direct anticoagulants of the heparin class and (v) antioxidants which are free radical scavengers.

The present invention further provides, by a second of its aspects, use of said protecting agents in the preparation of a contrast medium.

The invention still further provides, by a third of its aspects, a method for protecting an individual against harmful effects of a CM, comprising administering to the individual, together with the contrast medium, also a protecting agent as defined above.

The non-steroidal anti-inflammatory drugs (NSAID) are compounds which have an anti-inflammatory, and in addition anti-pyretic and analgesic activities. Examples of NSAID are:

- Aniline derivatives such as paracetamol, phenacetin;
- Anthranilic derivatives such as flufanamic acid, mefenamic acid;
- Phenyl alkanoic derivatives such as fenoprofen, flurbiprofen, ibuprofen, ketoprofen;
- Pyrazole derivatives such as amidopyrine, dipyron, phenazone, phenylbutazone;

- Salicylic acid derivatives such as aspirin, salicylic acid, choline salicylate.

Examples of agents which induce cells to generate nitrous oxide (NO) are nitroglycerine, nitroprusside, isosorbide dinitrate and others.

Polysaccharides which act in sealing the intercellular spaces between endothelial cells are agents capable of forming a coating over the walls of blood vessels and of forming a barrier between the interior of the blood vessel and the cells. An example of such an agent is dextran.

Examples of direct anti-coagulants of the heparin class are: heparin, low molecular weight heparins (such as innoxaprine) and the like.

Anti-oxidants which are free radical scavengers are useful particularly where the CA is of a kind that is capable of inducing the formation of free radicals which cause cellular damage. Examples for such agents are glutathione, tocopherols and the like.

The invention will now be illustrated in the following examples with occasional reference to the annexed drawings:

## BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows the effect of CA (added at  $t_1$  and  $t_2$ ) on intracellular calcium endothelial cells in vitro.

Fig. 2 demonstrates the effect of the combination of PA and CA upon the intracellular calcium (CA and PA were added together at  $t_1$  then washed and then CA was added separately at  $t_2$ ).

## EXAMPLES

### Cell Culture Preparation

Human umbilical vascular endothelial cells (HUVEC) CRL-1730 (American Type Culture Collect) were cultured according to known techniques. Briefly, HUVEC were grown in F-12K medium (Irving Sci. USA) containing 30  $\mu\text{g}/\text{ml}$  Endothelial Cell Growth Factor, 100  $\mu\text{g}/\text{ml}$  heparin, 10% fetal calf serum and 5% neonatal calf serum. Cells were seeded at an initial density of  $1-2 \times 10^5$  cells/ $\text{cm}^2$  and kept at  $37^\circ\text{C}$  under an atmosphere of 5%  $\text{CO}_2$  in air. To all such mediums, 4.5 mg/ml glutamine, 50 units/ml penicillin,



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200 µg/ml of streptomycin and 1250 units/ml nystatin were added. Media were changed twice weekly and cells were grown for about 7-10 days until confluence. Confluent cells were trypsinized and experiments were performed when trypan blue was excluded from 95% or more of the cells.

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**Measuring Techniques****Image analysis procedures and calibration**

[Ca<sup>+2</sup>] analysis was performed by using an Applied Imaging Magical system (TARDIS version 7.3). Cover slips with attached endothelial cells in the epifluorescence mode (oil-immersion objective, X 40) and equipped with a xenon lamp. Intracellular calcium measurements were assayed by ratio imaging of FURA-2/AM (excitation, 340 and 380 nm; emission, 515 nm). A temperature-controlled cell perfusion chamber (Applied imaging) was used to keep cells at a temperature of 32°C. All solutions were maintained at 32°C in a temperature controlled bath. Single cell images (10-40 cells) were collected by an intensified video camera (Photonic Science). The video signal was averaged over 4 video frames and averaged image pairs (340 and 380 nm) were captured every 5-15 seconds. Images were digitized at 256 x 256 pixels and averaged after subtracting the background.

Intracellular calcium calibration: Fura-2/AM signals were calibrated by addition of Ionomycin (5 µM) to cells kept in a 2 mM Ca<sup>+2</sup> solution to obtain maximum fluorescence. An addition of 10 mM of [ethylene-bis- (oxyethylenenitrilo)]-tetraacetic acid) (EGTA) followed in order to adjust the pH to > 8.5 in order to obtain the minimal fluorescence. The intracellular calcium was calculated as previously described employing a Ca<sup>+2</sup>-Fura 2 dissociation constant of 224 nM.

**Tetrazolium salt assay**

The 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) colorimetric reaction is based on the uptake of tetrazolium salt exclusively by live cells and its reduction to a soluble violet (formazan) compound. Absorbance of formazan is proportional to the amount of active

mitochondrial enzyme succinate-dehydrogenase of the cells and consequently to cell viability. Both endothelial or kidney cells were seeded at 30000-40000 cells/well in 96-well microliter wells (Corning) and grown until confluence. The measurements were performed after statistically testing the assumption that endothelial cells as well as the kidney cell line display contact inhibited cell growth, therefore yielding about equal cell numbers in each well. The absorbance spectrum of MTT was determined by a diode array spectrophotometer (Hewlett Packard, 8452A). MTT exhibited peak absorbance at 560 nm and minimal readings beyond 620 nm, as previously shown. A microplate reader (Thermomax, Molecular Devices) was used to read absorbance at 550 nm with background subtraction at a reference absorbance of 650 nm at 25 °C.

The experiments employed confluent cells which were loaded with 0.15 µg/ml of MTT and dark incubated at 37°C. Four hours later the medium was removed by plate flipping and 100 µg/ml DMSO were added to each well in order to solubilize the formazan crystals. Results are expressed in percentages as compared to control values of untreated cells and each result consisted of four to six repeated measurements in at least two different experiments. HUVEC cells were exposed to CM for either 4 or 24 hours prior to optical density readings.

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#### PRIOR ART REFERENCE EXPERIMENTS

The cytotoxic effect of CA materials on these cells was hitherto evaluated by Dascalu and Peer (1994)<sup>(11)</sup>, in a series of experiments wherein the viability of the cells was determined in the presence or absence of CA. This evaluation was done by determining the tetrazolium salt (MTT) which was reduced to violet formazan as will be further elaborated. This reaction required the activity of mitochondrial dehydrogenase and thus is indicative of cell viability. The results presented there show that both endothelial and kidney cells are damaged when exposed to CA for 4 hours.

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**Example 1**

The effect of using PA in conjunction with CA materials was evaluated by determining the cytotoxic effect and intracellular calcium levels in endothelial cells with or without PA.

5           The effect of CA (in this case Telebrix™, (Guerbet, France)) can be seen in Fig. 1. As can be seen, upon addition of 35  $\mu$ l of CA into 1 ml of hepes-buffered saline at  $t_1$ , there is an immediate increase in the intracellular calcium level. The calcium level increase also occurs upon re-addition of the same CA at  $t_2$ .

10           When a CA as described above was administered together with indomethacin, as shown in Fig. 2 (at  $t_1$ ), there was no or negligible increase in the intracellular calcium level. Upon re-administration of the CA, at  $t_2$ , after the CA and indomethacin were washed out, there was a marked increase in the intracellular calcium level. This demonstrates the protective effect  
15           which indomethacin has on such harmful effects of the CA.

**Example 2**

Endothelial cell viability was tested upon 4 hours of exposure to CA with or without a protecting agent (PA). The results are shown in the  
20           following Table 1:

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Table 1

CA*	PA**	CA only	CA+PA
CA1	A	142 ± 17	199 ± 40
CA1	A	228 ± 26	265 ± 29
CA1	A	407 ± 52	452 ± 43
CA1	A	707 ± 53	768 ± 16
CA2	A	678 ± 94	835 ± 62
CA2	A	315 ± 38	369 ± 38
CA3	A	774 ± 103	929 ± 64
CA3	A	602 ± 73	752 ± 134
CA3	B	884 ± 99	1059 ± 205
CA2	B	355 ± 18	402 ± 56
CA4	B	124 ± 24	228 ± 62
CA2	C	678 ± 94	800 ± 85
CA4	C	244 ± 23	292 ± 31
CA1	D	856 ± 69	934 ± 55
CA2	D	678 ± 94	825 ± 65
CA1	E	707 ± 53	812 ± 44
CA1	E	583 ± 55	769 ± 32
CA1	E	228 ± 26	297 ± 41
CA3	E	602 ± 73	802 ± 141

\* CA1 = Ultravist, Schering, AG, Germany  
 CA2 = Hexabrix-320, Guerbet, France  
 CA3 = Omnipaque, Schering AG, Germany  
 CA4 = Conray-60, Malinckrodt, U.S.A.

\*\* A = Indomethacin (50 micromolar)  
 B = Heparin (100 units/ml)  
 C = Dextran (50 microgram/ml)  
 D = Nitroprusside (50 micromolar)  
 E = Glutathione (2 mM).

The average and the standard deviations of the absolute optical density readings of formazan absorption of light are presented in the above Table. The results given, reflect the mitochondrial activity of the tested cells, and serve as an indicative tool to evaluate the cells viability.

**CLAIMS:**

1. A contrast medium, comprising an effective amount of a contrast agent, and an effective amount of a protecting agent being an agent active in protecting against possible damaging effects of the contrast medium, said protecting agent being one or more members selected from the group consisting of:
  - (i) non-steroidal anti-inflammatory drugs (NSAID);
  - (ii) agents which induce cells to generate nitrous oxides (NO);
  - 10 (iii) polysaccharides capable of sealing the intercellular spaces which exist between endothelial cells;
  - (iv) direct anticoagulants of the heparin class; and
  - (v) antioxidants which are free radical scavengers.
- 15 2. Use of a contrast medium in combination with a protecting agent selected from the group consisting of:
  - (i) non-steroidal anti-inflammatory drugs (NSAID);
  - (ii) agents which induce cells to generate nitrous oxides (NO);
  - (iii) polysaccharides capable of sealing the intercellular spaces which exist  
20 between endothelial cells;
  - (iv) direct anticoagulants of the heparin class; and
  - (v) antioxidants which are free radical scavengers.
- 25 3. A method for protecting an individual against harmful effects of contrast media in medical procedure involving injection to said individual of a contrast medium, comprising administering to the individual a contrast medium in combination with a protecting agent being a member of the group consisting of:
  - (i) non-steroidal anti-inflammatory drugs (NSAID);
  - 30 (ii) agents which induce cells to generate nitrous oxides (NO);
  - (iii) polysaccharides capable of sealing the intercellular spaces which exist between endothelial cells;

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- (iv) direct anticoagulants of the heparin class; and
- (v) antioxidants which are free radical scavengers.

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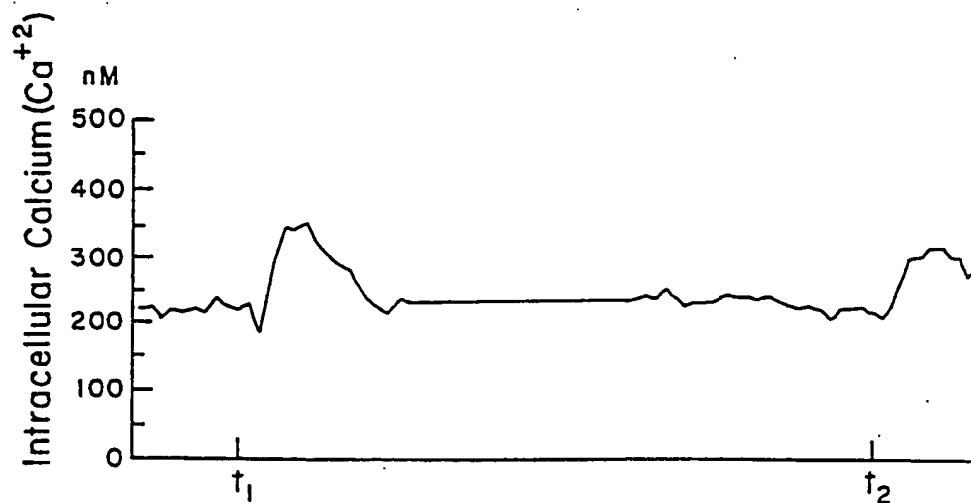


Fig.1

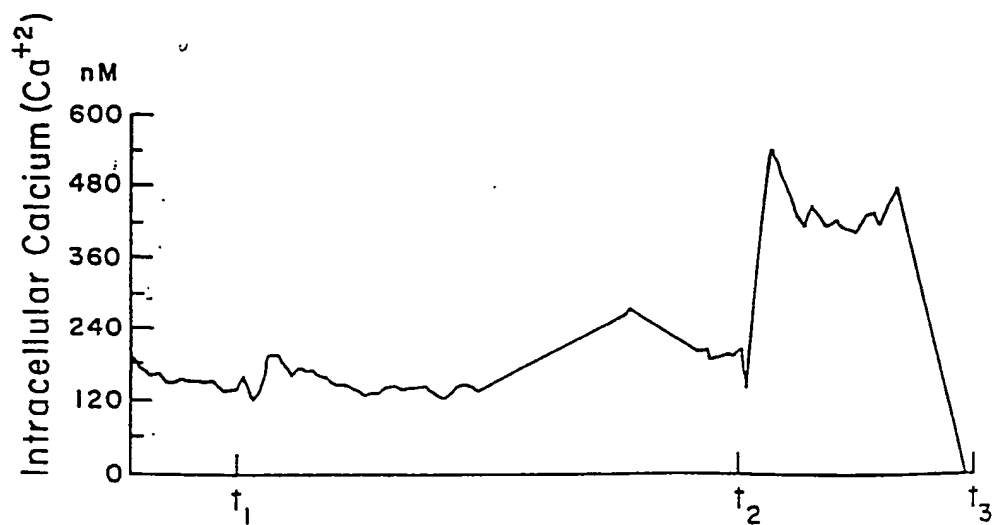


Fig.2

# INTERNATIONAL SEARCH REPORT

International application No.  
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## A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : A61K 49/00; G01N 31/00, 33/48

US CL : 424/9.3; 536/21, 123.1; 252/397

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## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/1.11, 1.65, 9.1, 9.3, 9.33, 9.4, 9.5; 536/21, 123.1; 252/397; 514/56; 554/2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, STN, MEDLINE, BIOSIS, CAPLUS, EMBASE

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US, A, RE 34,077 (SEGALL ET AL) 22 September 1922, see entire document, especially, column 5, lines 27-53; columns 6-7, lines 65-68 and 1-10, respectively; column 18, Example 8.	1-3
X	US, A, 5,199,951 (SPEARS) 06 April 1993, see entire document, especially, columns 7-8, lines 7-50 and 8-68, respectively.	1-3
X	US, A, 5,334,370 (JOSEF ET AL) 02 August 1994, see entire document, especially, columns 2-3, lines 53-68 and 1-2, respectively; column 22, claims 11-13.	1-3

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be of particular relevance	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"G" document member of the same patent family
"O" document referring to an oral disclosure, use, exhibition or other means	
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## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	British Medical Bulletin, Volume 50, Number 4, issued October 1994, VERSTRAETE, "Primary and secondary prevention of arterial thromboembolism", pages 946-965.	1-3

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(54) Title: PODOPHYLLOTOXINS AS ANTIPROLIFERATIVE AGENTS

(57) Abstract: The invention relates to podophyllotoxins, uses thereof and methods of their production.

### Podophyllotoxin

Lignans, such as podophyllotoxin and its metabolites/precursors are part of the phenylpropanoid pathway and are widely distributed throughout the plant kingdom. Some of them, in particular podophyllotoxin itself, are known to have anti cancer, antifungal and/or antimicrobial properties. Podophyllotoxin was first extracted from may apple (*Podophyllum peltatum*) and from *Linum* species, such as *Linum album*, *Linum flavum* and *Linum nodiflorum* as a resin and was used by physicians in the southern parts of the USA in the late 19<sup>th</sup> century for the treatment of genital warts, the latter being associated with some parts of cancer through their etiology by human papillomavirus (HPV). Podophyllotoxin has gained particular interest as the parent molecule of chemotherapeutic drugs such as etoposide, teniposide and etopophos, which are inhibitors of topoisomerase II. At present, the demand for podophyllotoxin outreaches the global supply by far, which has become insufficient due to slow growth and overcollection of the wild plants. In order to compensate for the limited supply of podophyllotoxin attempts have been undertaken to cultivate cells of *Podophyllum peltatum* and *Linum album* which, to some extent have been successful. The semisynthetic derivatives, etoposide and teniposide are widely used important anticancer drugs, but they have several limitations, such as poor water solubility, metabolic inactivation and the development of drug resistance. To overcome these limitation derivatives of podophyllotoxin have been synthesised in many laboratories (Yin et al., *acta pharm. Sinica* 1993, 28, 758-761, Wang et al., *acta chem. Sinica* 1992, 50, 698-701, Chang et al., *J. med. chem.* 1994, 37, 446-442, Pelter et al., *J. nat. prod.* 1994, 57, 1598-1602). None of these, however, have proved a substantial amelioration in terms of both efficacy as well as the prevention of side effects.

Etoposide is a widely used highly effective anti cancer drug against a broad spectrum of tumors including paediatric cancers such as acute lymphatic lymphomas, rhabdomyosarcomas and neuroblastomas as well as in most common adult cancers. It is also used in bone marrow

transplantation conditioning regimens. However, the therapeutic use of etoposide is limited by its toxicity involving mainly myelosuppression.

One of the major challenges for successful chemotherapy in cancer in general and high risk leukemia in particular is to overcome multidrug resistance. The majority of patients initially respond to treatment with combinations of various chemotherapeutic agents. However, poly-chemotherapy may induce multidrug resistant (MDR) cell clones which continue to proliferate in the presence of cytotoxic agents (Dalton WS. Mechanism of drug resistance in hematologic malignancies *Semin Hematol.* 1997;34:3-8). The reduction of chemosensitivity in such cell clones would require the administration of cytostatic agents in quantities exceeding the maximum tolerated dose in vivo. One of the best-characterized resistance mechanisms in leukemias and carcinomas is the drug extrusion mediated by p-glycoprotein, the product of the multidrug resistance-1 gene (MDR-1), which has been shown to be associated with poor outcome (Hunault M, Zhou D, Delmer A, et al. Multidrug resistance gene expression in acute myeloid leukemia: major prognosis significance for in vivo drug resistance to induction treatment. *Ann Hematol.* 1997;74:65-71; Marie JP, Zhou DC, Gurbuxani S, Legrand O, Zitoun R. MDR1/P-glycoprotein in haematological neoplasms. *Eur J Cancer.* 1996;32A:1034-1038.).

A variety of strategies have been developed to avoid or circumvent drug resistance since the introduction of poly-chemotherapy. Efforts to overcome established drug resistance in patients focus on (i) scheduling, i.e. prolonged low dose therapy such as antifolates in relapsed leukemia or short term high dose administration, e.g. antifolates with subsequent folate-rescue in osteosarcoma, (ii) combination therapy with chemical sensitizers such as MDR-1 inhibitors in the case of MDR-mediated resistance and (iii) combination of chemotherapy with non-chemical sensitizers such as radiotherapy, hyperthermia or hyperbaric oxygen (Dalton WS. Mechanisms of drug resistance in hematologic malignancies. *Semin Hematol.* 1997;34:3-8; Joel SP, Slevin ML. Schedule-dependent topoisomerase II-inhibiting drugs.

Cancer Chemother Pharmacol. 1994;34 Suppl:S84-S88; Ishikawa T, Kuo MT, Furuta K, Suzuki M. The human multidrug resistance-associated protein (MRP) gene family: from biological function to drug molecular design. Clin Chem Lab Med. 2000;38:893-897.)

Only few attempts have been made to directly modify the cytostatic agent in order to find analogues that actively evade drug resistance mechanisms such as a deaminated doxorubicin analogue (Solary E, Ling YH, Perez-Soler R, Priebe W, Pommier Y. Hydroxyrubicin, a deaminated derivative of doxorubicin, inhibits mammalian DNA topoisomerase II and partially circumvents multidrug resistance. Int J Cancer. 1994;58:85-94.), and beta-amino derivatives of etoposide (Zhang YL, Guo X, Cheng YC, Lee KH. Antitumor agents. 148. Synthesis and biological evaluation of novel 4 beta-amino derivatives of etoposide with better pharmacological profiles. J Med Chem. 1994;37:446-452; Zhang YL, Shen YC, Wang ZQ, et al. Antitumor agents, 130, Novel 4 beta-arylamino derivatives of 3',4'-didemethoxy-3',4'-dioxo-4-deoxypodophyllotoxin as potent inhibitors of human DNA topoisomerase II. J Nat Prod. 1992;55:1100-1111.). Thus, possible solutions to MDR related failures include the rational design of drugs which are not affected by MDR mechanisms and exhibit reduced systemic toxicity and increased anti-tumor potency.

Resistance against etoposide occurs at distinct cellular levels, involving downregulation of the target enzyme topoisomerase II, downregulation of either pro- or upregulation of anti-apoptotic mechanisms such as bcl-2, and increased metabolism and/or extrusion of the drug from the cell mediated by transport systems. The induction of such transport systems frequently leads to cross resistance against other cytostatic agents, as observed for MDR-1, MRP, or LRP mediated multidrug resistance (Gottesman MM. How cancer cells evade chemotherapy: sixteenth Richard and Hinda Rosenthal Foundation Award Lecture. Cancer Res. 1993;53:747-754; Borst P, Evers R, Kool M, Wijnholds J. A family of drug transporters: the multidrug resistance-associated proteins. J Natl Cancer Inst. 2000;92:1295-1302; Borst P,

Evers R, Kool M, Wijnholds J. The multidrug resistance protein family. *Biochim Biophys Acta*. 1999;1461:347-357.) A major mechanism of drug resistance, documented to occur in hematologic malignancies, is the overexpression of the MDR-1 gene product, P-glycoprotein. Therefore, attempts to overcome transport-system mediated drug resistance focused thus far mainly on modulation of MDR-1 expression (Liu C, Qureshi IA, Ding X, et al. Modulation of multidrug resistance gene (*mdr-1*) with antisense oligodeoxynucleotides. *Clin Sci (Colch)*. 1996;91:93-98.) or coadministration of MDR-1 inhibitors such as cyclosporin (Sonneveld P, Durie BG, Lokhorst HM, et al. Modulation of multidrug-resistant multiple myeloma by cyclosporin. The Leukaemia Group of the EORTC and the HOVON. *Lancet*. 1992;340:255-259.), verapamil (Joly P, Lallemand A, Oum'Hamed Z, Trentesaux C, Idoine O, Desplaces A. Effects of verapamil and S9788 on MDR-1 mRNA expression studied by in situ hybridization. *Anticancer Res*. 1996;16:3609-3614.) or valspodar (Tai HL. Technology evaluation: Valspodar, Novartis AG. *Curr Opin Mol Ther*. 2000;2:459-467.) all of which revealed limited efficacy in vitro and in vivo. These membrane transport proteins extrude a surprisingly wide range of substrates with entirely different structures, possibly due to the fact that common metabolites such as glucuronide, glutathione or sulfate rather than the different drugs are specifically recognized (Zhu BT. A novel hypothesis for the mechanism of action of P-glycoprotein as a multidrug transporter. *Mol Carcinog*. 1999;25:1-13.). Therefore, these resistance mechanisms resemble the ubiquitin system, where a plethora of entirely different proteins are "tagged" with ubiquitins in order to be first recognized and then degraded by proteasomes. Thus drug modifications that interfere with molecular "tagging" result in new molecules which may not be cleared from multidrug resistant tumor cells.

Some attempts have been undertaken to synthesise derivatives of etoposide which should allow a more specific targeting of etoposide to the target tissues. EP0423747 reports the synthesis of glycosyl-etoposide-prodrugs which by the action of tumor-specific enzyme

conjugates can be cleaved into the effective etoposide drug and a glycosylic residue, whereby, because of the tumor specific enzyme conjugates, the drug is activated only at its preferred site of action. (cf. also US4975278). Shabat et al. (PNAS, vol. 98, 13, 7528-7533) describe the synthesis of an antibody-prodrug system based on etoposide wherein the 4'-phenolic OH-group was masked by an aldol carbamate compound. Such a prodrug alone, however did not show any anti-tumour activity, unless it was combined with a catalytic antibody 38C2, which activates the prodrug to yield etoposide. The prodrugs described in Shabat et al. are only activated by retro-Michael/retro-aldol reactions which do not occur in nature. Only artificial enzymes, like the 38C2 catalytic antibody can catalyse the conversion. The handling and the use of such a prodrug is made more difficult by the additional requirement of the co-application of a catalytic antibody which catalyses the conversion into the active drug. Therefore, none of the above mentioned derivative prodrugs have proven particularly useful in the treatment of the above mentioned cancers.

Prodrugs of various antitumor agents have been synthesised in order to improve their bio-availability, pharmacokinetics and aqueous solubility. WO99/30561 describes a nucleotide-based prodrug wherein the release and activation of the drug component arises from the hydrolysis of the junctional ester bond joining the nucleotide component to the drug component.

US4,975,278 describes a method for the delivery of cytotoxic drugs to tumor cells by the administration of a tumor-specific antibody-enzyme conjugate that binds to the tumor cells, and the additional administration of a prodrug that is converted at the tumor side, in the presence of the antibody-bound enzyme, to an active cytotoxic drug. This concept has been used in conjunction with etoposide-4'-phosphate or 7-(2'-aminoethyl phosphate)-mitomycin. Again this has been of limited utility so far.

WO94/13324 describes the conversion of drugs into prodrugs by converting their corresponding functional groups into 1-O-alkyl-, 1-O-acyl-, 1-S-acyl- and 1-S-alkyl-sn-glycero-3-phosphate derivatives. None of the reported prodrugs of WO94/13324 have proven particularly effective in the treatment of cancers.

WO98/13059 reports on prodrugs comprising an amino-terminal capped peptide that is a substrate for a peptidohydrolase located on the surface of a metastatic cell. The anticancer drug typically used for that purpose is doxorubicine, taxol, camptothecin, mitomycin C or esperamycin. The peptidohydrolase that hydrolyses the substrate of the prodrug is typically cathepsin B.

US-Patent No. 5,977,065 describes prodrugs of actinomycine D, doxorubicin, mitomycin C or nitrogen mustard arising from a reaction with 4-nitrobenzylchloroformate.

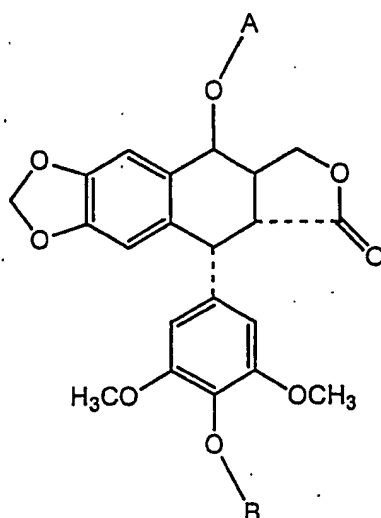
European Patent Application EP 0320988 to Bristol-Myers Company discloses 4'-esters, 4'-carbonates and 4'-carbamates of 4'-demethylepipodophyllotoxin glucosides for which a certain antitumor activity in animals is reported. The compounds disclosed in EP 0320988 are not capable of overcoming multidrug resistance.

Nicolaou (Nature 1993, 364, 464) and Niethammer et al. (Bioconj. Chem. 2001, 3, 414) report on a paclitaxel prodrug blocked at the C7 hydroxyl group with a dihydroxy propyl sidechain which can be hydrolytically cleaved by a pH-dependent, slow-release mechanism. The resulting prodrug showed some advantages in relation to the parent drug in that it was more water soluble and could be used at a 3-fold higher maximum tolerated dose (MTD). Paclitaxel is an anticancer agent which is particularly used in breast, lung and ovarian cancers. It is known to promote the irreversible polymerisation of tubulin thereby disrupting the cell division by cell cycle arrest in the premitotic G2 phase. A second cytotoxic mechanism of paclitaxel is to assist the induction of TNF alpha, an event unrelated to the polymerisation of



microtubules. The paclitaxel prodrugs described in the aforementioned publications are unstable in aqueous solution and hydrolyse spontaneously. Therefore the utility of the prodrugs reported in Niethammer et al. is very limited. So far, no prodrug has been reported in relation to etoposide which showed substantially improved efficacy and highly reduced side effects in comparison to the parent molecule. Accordingly it has been an object of the invention to provide for a prodrug of podophyllotoxins which substantially reduces adverse reactions when administered to patient. It has also been an object of the present invention to provide for prodrugs of podophyllotoxins that are stable in aqueous solutions, yet do not require the application of catalytic antibodies for their conversion into the active drug. It has furthermore been an object to provide for prodrugs of podophyllotoxins that allow for a slow release of the drug at the intended side of action, i.e. a tumor. It has also been an object of the present invention to provide for prodrugs of podophyllotoxins that are capable of overcoming multidrug resistance commonly encountered with the parent molecule of the drug. Furthermore it has been an object to provide for a method of preparing such prodrugs as well as a pharmaceutical composition comprising such prodrugs as well as providing potential uses of such prodrugs and such pharmaceutical composition.

This object is solved by a podophyllotoxin represented by formula I



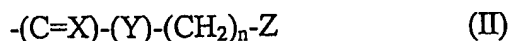
(I)

wherein A is H or is selected from the group comprising carbohydrates, polyols,  $\text{-(CH}_2\text{-CH(OH)-)}_p\text{-H}$ ,  $\text{H-(CH}_2\text{-CH(OH)-)}_p\text{-}$ , ethylene glycol, propylene glycol, glycerol, penta-

erythritol, polyethyleneglycol and compounds, as represented by formula III below,

wherein  $p$  is an integer from 2 to 100, and

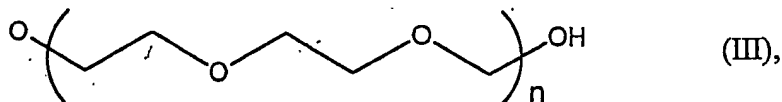
wherein B is represented by formula II



wherein X is selected from the group comprising O, S and NR'', Y is selected from the group comprising O, S, and NR'', wherein R'' = alkyl, aryl or H,

n is an integer of from 0 to 6; and

Z is a polyhydroxyalkyl group selected from the group comprising ethylene glycol, propylene glycol, glycerol, pentaerythritol, polyethyleneglycol and compounds represented by formula III



or Z is a polyhydroxyalkyl group, as defined above, which additionally has a dioxolane group attached,

or Z is a dioxolane group,

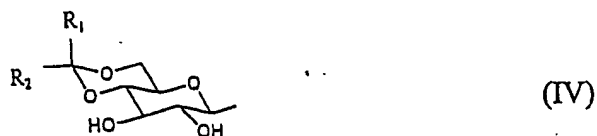
or Z is selected from the group comprising targetting moieties for mammalian receptors, antibodies, steroids, transferrin, proteins and peptides having tumor cell associated receptor finding function,

In one embodiment, the dioxolane group is selected from the group comprising 2,2-dialkyl-1,3-dioxolane, wherein each alkyl at the 2-position is independently selected from the group comprising unsubstituted and substituted methyl, ethyl, propyl, butyl, pentyl and hexyl.

In one embodiment, Z is a targeting moiety for a mammalian surface membrane receptor or for a mammalian nuclear receptor, or Z is an antibody, steroid, transferrin, protein or peptide having tumor cell associated receptor binding function, wherein, preferably, Z is selected

from the group comprising steroids, growth factor receptor inhibiting proteins, peptides and non-peptide mimetics.

In one embodiment, A is selected from the group comprising compounds as represented by formula IV



wherein R<sub>1</sub> and R<sub>2</sub> are each C<sub>1</sub>-C<sub>10</sub> alkyl; or R<sub>1</sub> and R<sub>2</sub> and the carbon to which they are attached represent C<sub>5</sub>-C<sub>6</sub> cycloalkyl; or R<sub>1</sub> is H and R<sub>2</sub> is selected from the group comprising C<sub>1-10</sub> alkyl, C<sub>2-10</sub> alkenyl, C<sub>3-5</sub> cycloalkyl, furyl, thienyl, C<sub>6-10</sub> aryl, and C<sub>7-14</sub> aralkyl.

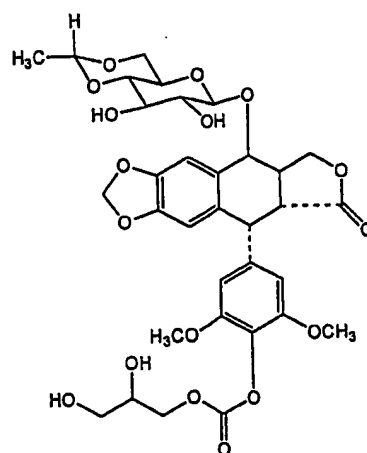
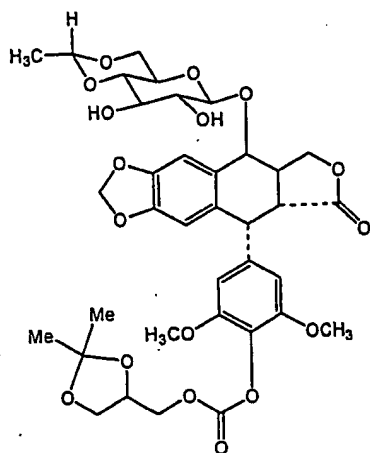
Preferably, R<sub>1</sub> is H and R<sub>2</sub> is methyl or thienyl.

In one embodiment, X is O and Y is O.

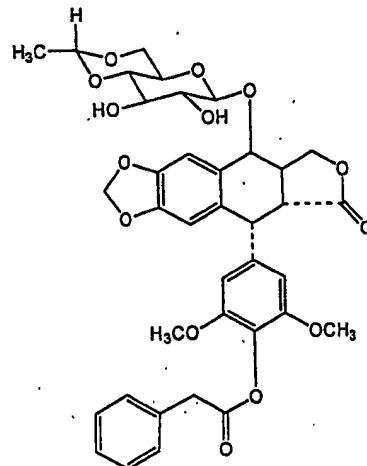
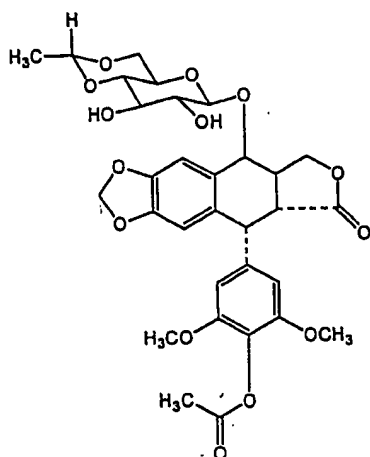
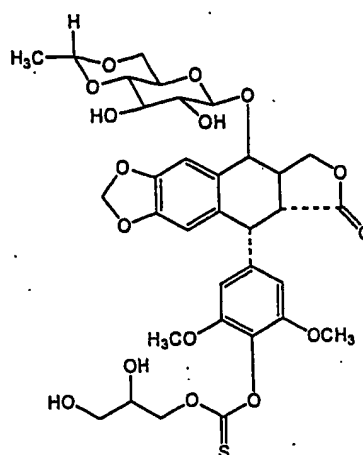
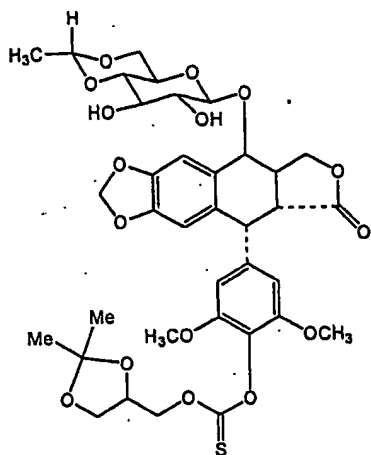
In another embodiment, X is O and Y is S.

In yet another embodiment, X is O and Y is NH.

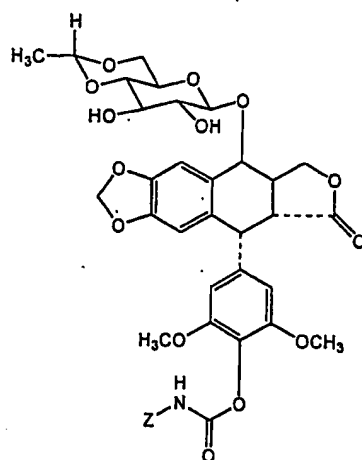
In a preferred embodiment, the podophyllotoxin is selected from the group comprising



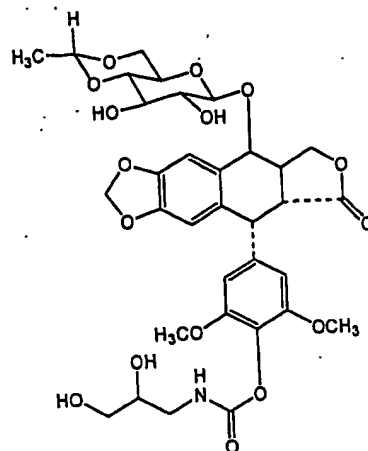
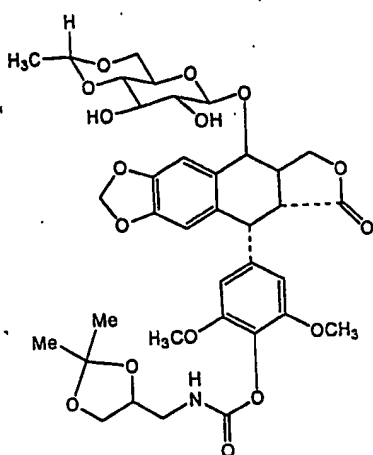
In another embodiment, it is selected from the group comprising



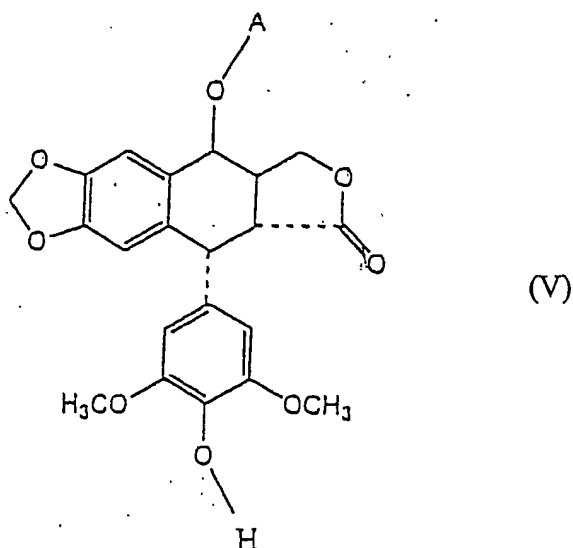
In yet another embodiment, it is selected from the group comprising



wherein Z is defined as in claim 1, wherein, preferably, the podophyllotoxin is selected from the group comprising



The objects of the present invention are also solved by a method of preparing a podophyllotoxin, characterized in that a compound as represented by formula V



A being as defined before,

is reacted with a haloformate  $W-(C=X)-(Y)-(CH_2)_n-Z$ , wherein X, Y, Z and n are as defined before, and W being Cl, F, Br or I,

or characterized in that a compound as represented by formula V is reacted with phosgene or trichloromethylchloroformate, to yield a 4'-phenol chloroformate intermediate,

said 4'-phenol chloroformate intermediate then being reacted with an alcohol or thiol of the formula  $ZYH$ , to yield the corresponding carbonate or thiocarbonate,  $Y=O$  or  $S$ , and Z being as defined before, or

said 4'-phenol chloroformate intermediate then being reacted with an amine of the formula  $HNR''Z$  to yield the corresponding carbamate,  $R''$  and Z being as defined before.

Preferably the compound as represented by formula V is reacted with a compound selected from the group comprising p-nitrophenyl soketalcarbonate, soketalchloroformate, and PEG-chloroformate.

In one embodiment of the method according to the present invention, the product resulting from the method as described before, is hydrolyzed.

The objects of the present invention are also solved by a pharmaceutical composition comprising a podophyllotoxin according to the present invention and a pharmaceutically acceptable carrier.

The objects of the present invention are also solved by a use of a podophyllotoxin according to the present invention or of a pharmaceutical composition according to the present invention for the manufacture of a medicament for the treatment of cell proliferative disorders, wherein, preferably, the cell proliferative disorder is in a child, an adolescent or an adult.

In order to create hydrolytically activated prodrugs of etoposide VP-16 as inactive precursor molecules which are activated by hydrolysis, derivatives of the 4' phenolic hydroxy group were synthesised. In previous studies it could be demonstrated that this 4' phenolic hydroxy group is very important for the cytotoxic activity of etoposide since a stable linker fused to that OH group decreased cytotoxicity by >3 logs (Shabat D, Lode HN, Pertl U, et al. In vivo activity in a catalytic antibody-prodrug system: Antibody catalyzed etoposide prodrug activation for selective chemotherapy. Proc Natl Acad Sci U S A. 2001;98:7528-7533.). The prodrugs reported by Shabat, however, were ineffective in conferring cytotoxicity and did not show any anti-tumour activity unless they were combined with the catalytic antibody 38C2 which activates those prodrugs to yield the active drug. The prodrugs reported in Shabat et al. are only activated by retro-Michael-retro-aldol reactions which do not occur in nature, such that catalytic antibody or specifically tailored enzymes have to be added that are capable of catalysing the conversion. In contrast thereto the prodrugs of the present invention are activated by a simple hydrolysis reaction, yet they are stable at a broad range of pH-values under normal physiological conditions.

In contrast to paclitaxel, podophyllotoxins, in particular etoposide, are topoisomerase II inhibitors, with a wide range of applications in human malignancies, including solid tumors and leucemias which are distinct from the field of applications of paclitaxel. For example etoposide is employed for the treatment of acute lymphatic leukemia, acute myeloid leukemia, neuroblastoma, and rhabdomyosarcoma. In contrast to the paclitaxel prodrugs that have been reported in the aforementioned publications (in particular Niethammer et al.) the prodrugs of the present invention are much more stable in aqueous solutions under physiological conditions, yet can be easily hydrolysed by appropriate pH changes. Without wishing to be bound by theory it is presently believed that one reason for that unexpected stability is the hydrophobic nature of the "southern" part of the molecule (i.e. at the phenol ring), in combination with the hydrophobic pocket which is formed at the 4' position by the two neighbouring methoxy groups at the 3' and 5' position, respectively.

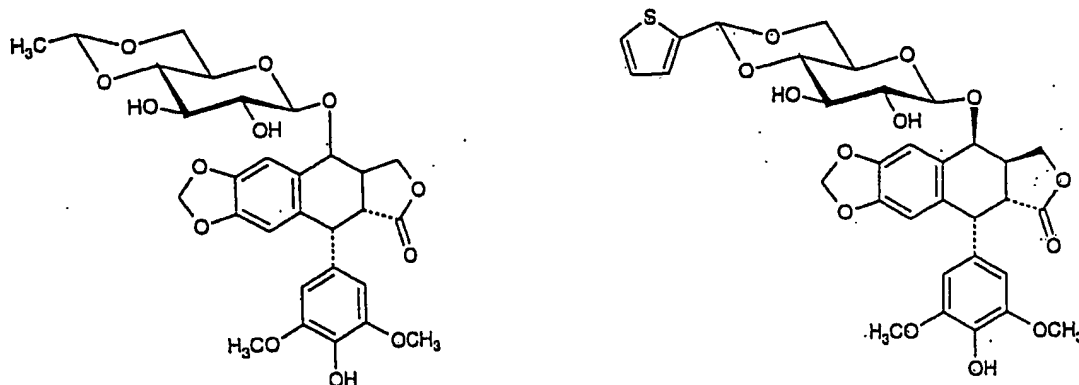
Therefore, the rational design of hydrolytically activated prodrugs of VP-16 involves the modification of the chemically reactive 4' phenolic hydroxy group which specifically tailors a desired effect. Depending on the nature of the chemical modification, the activation of the prodrug can be defined to occur at a desired pH dependent rate. The prodrugs of the present invention are stable under a wide range of pH values, yet can be activated by an appropriate change of pH, as well as under physiological conditions in the presence of naturally occurring enzymes, such as for example carboxyl esterases. This will be shown in more detail below.

Therefore it was surprisingly found that by rational design, hydrolytically activated prodrugs of etoposide, that retain full anti-tumor activity against multidrug resistant tumor cells in vitro and in vivo, can be produced.

As used herein the term "proteins and peptides having tumor cell associated receptor finding function" is meant to designate any protein or peptide that is capable of binding a receptor that is associated with tumor cells.

"Targeting moieties for mammalian receptors" are groups that are capable of binding to mammalian receptors. "Growth factor receptor inhibiting proteins, peptides and non-peptide mimetics" is meant to designate any protein, peptide or non-peptide small molecule compound that is capable of inhibiting a growth factor receptor by any mechanism, in particular

by binding thereto. "Etoposide and teniposide" is meant to designate compounds of the formula



, and any protonated form thereof. The term "functionality" is meant to designate any chemical moiety allowing the molecule to which such moiety is attached, to undergo a chemical reaction.

Reference is now made to the figures, wherein

figure 1 shows a scheme of synthesis of ProVP-16I and II,

figure 2 shows the conversion of ProVP-16I to ProVP-16II and VP-16,

figure 3 shows cytotoxicity profiles for ProVP-16I and II compared to VP-16,

figure 4 shows the effect of Pro VP-16I and II on multidrug resistant MOV-3 cells,

figure 5 shows the induction of apoptosis by ProVP-16I and II in resistant cells, and

figure 6 shows the toxicity and anti-tumour response following ProVP-16II therapy in mice.

In more detail, the figures are as follows:

Figure 1 shows a Scheme of synthesis and activation of ProVP-16 I and II. The synthesis of ProVP-16 I involves the reaction of solketal chloroformate and VP-16 as described in Materials and Methods. Compound ProVP-16 II is synthesized from ProVP-16 I by acid hydrolysis with the elimination of 2,2 dihydroxypropane. The activation of ProVP-16 II to VP-16 occurs with the elimination of glycerol and carbon dioxide.

Figure 2 shows the Conversion of ProVP-16 I to ProVP-16 II and VP-16. ProVP-16 I was incubated in THF / 2% HCl and samples were periodically analyzed by HPLC at the indicated time points (A). Hydrolytic activation of ProVP-16 II was determined in phosphate buffered saline (PBS) at the pH levels indicated (B). ProVP-16 I (3mM) was incubated in PBS solu-



tions and samples were periodically analyzed by HPLC. The per cent conversion was calculated from areas under the curve determined by peak integration.

Figure 3 shows Cytotoxicity profiles for ProVP-16 I and II compared to VP-16. The cytotoxic effect of ProVP-16 I and II was evaluated against a panel of cell lines by triplicate determinations of  $IC_{50}$  concentrations for both prodrugs against each cell line (A). The cytotoxicity mediated by the prodrugs relative to VP-16 was calculated according to  $IC_{50} \text{ VP-16} \div IC_{50} \text{ ProVP-16 I or II}$ . Bars represent mean values  $\pm$  SD. The differences between ProVP-16 I or II and VP-16 were statistically significant ( $p < 0.01$ ) for all cell lines except NXS2. Stars indicate cell lines with amplified MDR-1 expression.

The slow release kinetics of cytotoxicity by hydrolytically activated ProVP-16 I was determined using Molt-3 cells (B).  $10^4$  cells per well were incubated with increasing concentrations ( $10^{-10}$  M to  $10^{-6}$  M) of ProVP-16 I and VP-16 in 96 well plates. At the time points indicated, cell viability was determined in triplicate by the XTT assay as described in Materials and Methods. Per cent cell viability was calculated from optical density measurements at 450 nm according to  $OD_{450} \text{ sample} \div OD_{450} \text{ untreated} \times 100\%$ . Results were plotted as a semilogarithmic function of drug concentration.

Figure 4 shows the Effect of ProVP-16 I and II on multidrug resistant MOV-3 cells. The newly generated VP-16 resistant MOV-3 cell line was analyzed for MDR-1 gene expression (A), resistance against VP-16 induced cytotoxicity (B) and cross resistance to MDR-1 drugs which are known substrates for p-glycoprotein (C). MDR-1 gene expression was determined by RT-PCR analysis on total RNA isolated from MOV-3 and Molt-3 cells, respectively (6A). Expression of GAPDH was used as a control for the integrity of the cDNA. Resistance of MOV-3 cells against VP-16 was calculated from  $IC_{50}$  concentrations according to  $IC_{50} \text{ MOV-3} \div IC_{50} \text{ Molt-3}$  ( $n=3$ ) and results compared to effects observed with ProVP-16 I and II (6B). Differential findings between Molt-3 and MOV-3 cells obtained with VP-16 were statistically significant ( $p < 0.001$ ) in contrast to ProVP-16 I and II ( $p > 0.05$ ). (6C) Cross-resistance of MOV-3 cells against MDR-1 drugs (doxorubicin, melphalan, vinblastine and paclitaxel) was calculated from  $IC_{50}$  values ( $n=3$ ) as described in 6B). Results for non-MDR-1 drugs (MTX, 5-FU, genistein, calicheamicin  $\theta$ , ProVP-16 I) are shown as controls. Differential findings for MDR-1 drugs between MOV-3 and Molt-3 cells were all statistically significant ( $p < 0.01$ ) in contrast to non-MDR-1 drugs ( $p > 0.05$ ).

Figure 5 shows the Induction of apoptosis by ProVP-16 I and II in resistant cells. The effect of ProVP-16 I and II ( $5 \times 10^{-7}$  M) on the cell cycle was analyzed in Molt-3 (A) and MOV-3 (B) cells and results were compared to VP-16 ( $5 \times 10^{-7}$  M). Cells were harvested at indicated time points (n=3), fixed, stained with propidium iodide and analyzed by FACS as described in Materials and Methods. Per cent cells in pre G1 (apoptotic cells) were calculated from DNA histograms.

Figure 6 shows the Toxicity and anti-tumor response following ProVP-16 II therapy in mice. A/J mice (n=6) were injected i.p. with ProVP-16 II (20 and 60 mg/kg) and VP-16 (20 mg/kg) on days 1, 3, 5, 7, 9, and 11 and with VP-16 (60 mg/kg) on days 1, 3 and 5 (A). The body weight was determined for each animal over time and calculated as per cent of total weight observed on day 0. An event as indicated by Kaplan Maier plots is defined by loss of body weight greater than 20%.

The anti-tumor effect of ProVP-16 II therapy was determined in a multidrug resistant xenograft model (B,C). SCID mice (n=7) were injected s.c. with  $5 \times 10^6$  MOV-3 cells and primary tumors of 250 mm<sup>3</sup> average size were established 55 days after inoculation. Treatment consisted of i.p. injections with Pro VP-16 II (45 and 15 mg/kg), VP-16 (15mg/kg) and solvent on days 55, 57, 59, 69, 71, 87 and 90 after tumor cell inoculation. Tumor growth was monitored by microcaliper measurements and tumor size was calculated as described in Materials and Methods (B). Differential findings between experimental groups of animals treated with ProVP-16 II (45 and 15 mg/kg) and control groups (solvent and VP-16) were statistically significant ( $p < 0.001$  after day 63) (B). The body weight of treated animals was determined over time and calculated as per cent of body weight on day 57 (C).

At the end of the treatment experiment, remaining s.c. tumors were removed and analyzed by RT-PCR for gene expression of MDR-1 except mice treated with 45 mg/kg with no residual tumor. Representative signals of one tumor of each group is depicted and compared to MDR-1 expressing VCR-100 cells used as a positive control. The presence of a 229 or 127 bp signals indicate expression of MDR-1.

The podophyllotoxins according to the present invention have surprisingly proven to be particularly effective in mediating cytotoxicity. Various derivatives of etoposide were synthesized and their activation mechanism via hydrolysis was clearly established (see examples that follow). Importantly, at physiological buffer conditions, the prodrugs remained stable. A prodrug of etoposide with a stable carbamate linker to block the 4' hydroxy group of

VP-16 had already been shown to be completely stable under physiological buffer conditions (Shabat D, Lode HN, Pertl U, et al. In vivo activity in a catalytic antibody-prodrug system: Antibody catalyzed etoposide prodrug activation for selective chemotherapy. *Proc Natl Acad Sci U S A*. 2001;98:7528-7533.). In contrast to the hydrolytically activated prodrugs of the present invention, exemplified by ProVP-16 I and II, the carbamate prodrug was designed to be activated only by catalysis of a retro-aldol retro-Michael reaction mediated by catalytic antibody 38C2, a reaction which does not occur in nature. It has to be stressed that this carbamate prodrug as opposed to the prodrugs of the present invention was ineffective in mediating cytotoxicity against all tumor cell lines investigated, thus demonstrating a crucial role for the 4' hydroxy group in VP-16 as a biologically relevant center capable of inducing cytotoxicity. The active center is also blocked in the prodrugs described here; however the prodrugs as comprised by the present invention with their hydrolytic activation mechanism do mediate cytotoxicity very effectively. The non-toxic nature of unconverted ProVP-16 I and II is shown by the slow release mechanism demonstrated in Molt-3 cells (Fig. 3) and by the absence of cytotoxic effects for the prodrugs up to 12h of incubation is in contrast to VP-16. A steady increase of cytotoxic activity of both VP-16 derivatives over time clearly demonstrated that the novel compounds of the present invention are initially stable and non-toxic, but then become activated inside the target cells. The slow release mechanism observed in vitro also accounts for the dramatically decreased systemic toxicity in mice, as demonstrated for ProVP-16 II being tolerated a greater than 3 fold increase in the maximum tolerated dose over the parental compound (Fig. 6).

Further characterization of the prodrugs according to the present invention revealed a higher potency in a number of cancer cell lines compared to VP-16. Thus, in cells with amplified MDR-1 gene expression (VCR 100, ADR 5000 and SW480) more than 3 log greater efficacy of the prodrugs according to the present invention was observed (Fig. 4). This is an increase rarely achieved by MDR-1 modulators (Dalton WS. Mechanisms of drug resistance in hematologic malignancies. *Semin Hematol*. 1997;34:3-8; Sonneveld P, Durie BG, Lokhorst HM, et al. Modulation of multidrug-resistant multiple myeloma by cyclosporin. The Leukaemia Group of the EORTC and the HOVON. *Lancet*. 1992;340:255-259; Joly P, Lallemand A, Oum'Hamed Z, Trentesaux C, Idoine O, Desplaces A. Effects of verapamil and S9788 on MDR-1 mRNA expression studied by in situ hybridization. *Anticancer Res*. 1996;16:3609-3614; Tai HL. Technology evaluation: Valspodar, Novartis AG. *Curr Opin Mol Ther*. 2000;2:459-467; Kang Y, Perry RR. Effect of alpha-interferon on P-glycoprotein expression

and function and on verapamil modulation of doxorubicin resistance. *Cancer Res.* 1994;54:2952-2958; Hofmann J, Gekeler V, Ise W, et al. Mechanism of action of dexniguldipine-HCl (B8509-035), a new potent modulator of multidrug resistance. *Biochem Pharmacol.* 1995;49:603-609.).

Furthermore, functional assays demonstrate that the prodrugs according to the present invention inhibit MDR-1 mediated substrate efflux. Consequently, it appears that the new prodrugs inhibit MDR-1 p-glycoprotein function accounting for the excellent activity against MDR-1 expressing cancer cell lines. Importantly, this dramatic in vitro effect also translated into a long lasting regression of established primary tumors in a drug resistant T-cell leukemia xenograft model in vivo (Fig. 6). In this model, MDR-1 gene expression is stably amplified with a 100x resistance against VP-16 in vitro (Fig. 4) resulting in the complete absence of a therapeutic effect by VP-16 at the maximum tolerated dose (Fig. 6). Interestingly, in such a challenging model exhibiting an artificially high drug resistance, treatment with the prodrugs according to the present invention can induce a dramatic anti-tumor response (Fig. 6). This is of particular importance since such a high MDR-1 amplification is rarely observed in patients following poly-chemotherapy even in relapsed malignancies (Beck J, Handgretinger R, Dopfer R, Klingebiel T, Niethammer D, Gekeler V. Expression of *mdr1*, *mrp*, topoisomerase II  $\alpha/\beta$ , and cyclin A in primary or relapsed states of acute lymphoblastic leukaemias. *Br J Haematol.* 1995;89:356-363; Beck JF, Bohnet B, Brugger D, et al. Expression analysis of protein kinase C isozymes and multidrug resistance associated genes in ovarian cancer cells. *Anticancer Res.* 1998;18:701-705.).

An important finding of this study is the highly effective anti-tumor response observed in multidrug resistant models with the prodrugs of the present invention in vitro and in vivo, suggesting that therapy with them will lead to a significant improvement over existing chemotherapy with ordinary podophyllotoxins.

The examples that follow are merely intended to illustrate, not to limit the scope of the present invention.

### Example 1

**Materials:** 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide (XTT), VP-16, solketal, organic solvents and phenazine methosulfate (PMS) were obtained from Sigma-Aldrich (Deisenhofen, Germany). Cell culture reagents, restriction enzymes and other molecular biology reagents were from Life Technologies (Karlsruhe, Germany).

**Cells:** Tumor cell lines were grown in RPMI, 10% FCS (Nalm-6, Reh, Molt-3, Jurkat, HL-60, K562, HeLa, CEM, A2780, SW480) or DMEM, 10% FCS (NXS2, SK-N-SH, HT-29) in the presence of 100 IU/ml penicillin/streptomycin (P/S) and propagated under standard tissue culture conditions (5% CO<sub>2</sub>, 37°C). All cell lines were obtained from ATCC, Rockville, MD except NXS2 which was previously described (Lode HN, Xiang R, Varki NM, Dolman CS, Gillies SD, Reisfeld RA. Targeted interleukin-2 therapy for spontaneous neuroblastoma metastases to bone marrow. *Journal of the National Cancer Institute*. 1997;89:1586-1594.) and VCR100, ADR5000 and A2780 which were kindly provided by James Beck, Greifswald, Germany (Beck J, Handgretinger R, Dopfer R, Klingebiel T, Niethammer D, Gekeler V. Expression of *mdr1*, *mrp*, topoisomerase II  $\alpha/\beta$ , and cyclin A in primary or relapsed states of acute lymphoblastic leukaemias. *Br J Haematol*. 1995;89:356-363; Beck JF, Bohnet B, Brugger D, et al. Expression analysis of protein kinase C isozymes and multi-drug resistance associated genes in ovarian cancer cells. *Anticancer Res*. 1998;18:701-705.). Molt-3 cells were used to generate an etoposide resistant subline MOV-3 by continuous exposure to increasing amounts of VP-16. After a time period of 6 months, MOV-3 cells were stable and propagated in the presence of 1  $\mu$ M VP-16 and used for further in vitro and in vivo experiments.

**Mice:** Female A/J mice and FOX CHASE<sup>TM</sup> C.B-17/lcrCrl-scid BR mice were obtained at 8 weeks of age from Charles River Laboratories, Sulzfeld, Germany. They were housed in the pathogen-free mouse colony at our institution in groups of 8 mice. Mice were fed ad libitum on standard mouse laboratory chow. Animal experiments were performed according to the German guide for the care and use of laboratory animals, i.e. "Tierschutzgesetz".

**Analytical chemistry of proetoposides:** The synthesis of proetoposides and analysis by HPLC has been previously reported Wrasidlo W, Schroeder U, Bernt K, et al. Synthesis, hydrolytic activation and cytotoxicity of etoposide prodrugs. *Bioorg Med Chem Lett*. 2002; 12: 557-560, which is hereby incorporated by reference in its entirety.

**RNA isolation, reverse transcription and PCR amplification:** Isolation of total cellular RNA, cDNA synthesis and RT-PCR-conditions were previously described (Lode HN, Xiang R, Varki NM, Dolman CS, Gillies SD, Reisfeld RA. Targeted interleukin-2 therapy for spontaneous neuroblastoma metastases to bone marrow. *Journal of the National Cancer Institute*. 1997;89:1586-1594.). The amplification of human MDR-1 was done with sense 5' GGA GAG ATC CTC ACC AAG CG 3' and antisense 5' GTT GCC AAC CAT AGA TGA AGG 3' for 35 cycles (15 s 96°C, 30 s 60°C, 90 s 72°C) leading to a 229bp fragment designated MDR-1. High sensitivity detection was achieved by nested amplification of 1.0µl MDR-1 after 21 cycles using sense 5' GCT CAG ACA GGA TGT GAG TT 3' and antisense 5' CTG GGT AAT TAC AGC AAG CC 3' for 30 cycles to create a 127bp fragment. The cDNA integrity was tested by amplification of glyceral-aldehyde-phosphate-dehydrogenase (GAPDH) with sense 5' CGG GAA GCT TGT GAT CAA TGG 3' and antisense 5' GGC AGT GAT GGC ATG GAC TG 3' for 25 cycles leading to a 358bp fragment. The specificity of all fragments was verified by sequencing.

**Functional JC-1 assay:** For staining, cells were washed twice and resuspended in PBS containing JC-1 monomer, as previously described (Legrand O, Perrot JY, Simonin G, Baudard M, Marie JP. JC-1: a very sensitive fluorescent probe to test Pgp activity in adult acute myeloid leukemia. *Blood*. 2001;97:502-508.). Briefly, 0.1 µM JC-1 monomer was incubated at 37°C for 15 minutes with  $5 \times 10^5$  cells/ml and incubated in the presence and absence of etoposide and Pro-VP-16 I and II. Samples incubated with 2µM cyclosporin A were used as positive controls (data not shown). Cell fluorescence was recorded using a FACSsort flow cytometer (Becton Dickinson) and JC-1 signals were detected on the FL1-channel (530 nm filter) for analysis of the dye monomer.

**Stable transfection of MDR-1:** The MDR-1 cDNA was kindly provided in a pUC based plasmid by C. Baum, University of Hamburg. For mammalian expression, the MDR-1 cDNA was cloned into the bicistronic eukaryotic expression plasmid pIRESpuro using NotI and BamHI restriction sites. pMDR-IRESpuro was transfected into Molt-3 cells by electroporation (960 µF, 250V). Stable transfectants were selected with 300 ng/ml puromycin. MDR-1 expression was determined by FACS-analysis (FACS-calibur, Becton Dickinson, Bedford, MA) using 1µg/ml MDR-1 specific mAb (C. Baum, University of Hamburg).

**Cytotoxicity assay:** Cytotoxicity was determined by the XTT tetrazolium/formazan assay as previously described (Scudiero DA, Shoemaker RH, Paull KD, et al. Evaluation of a soluble tetrazolium/formazan assay for cell growth and drug sensitivity in culture using hu-

man and other tumor cell lines. Cancer Res. 1988;48:4827-4833.). Briefly, cells were seeded in 96 flat bottom well plates at a density of  $10^4$ /well in 100  $\mu$ l media and exposed to drug concentrations ranging from  $10^{-4}$  to  $10^{-12}$  M. At the indicated time points (6-72hr), cell viability was assessed by adding 50 $\mu$ l XTT-reagent (1mg/ml in serum free RPMI) activated with 0.2% v/v PMS (1.53 mg/ml in PBS) incubated at 37°C for 4h. Plates were analyzed in a Thermo-max (Molecular Devices) micro plate reader at 450 nm. OD values were plotted as a function of drug concentration and the curves were integrated using the softmax software to obtain the IC<sub>50</sub> concentration values.

**Cell cycle analysis:** Distinct phases of the cell cycle were determined in a standard assay using propidium iodide. Briefly, cells were seeded in 24 well plates ( $10^5$  /well) and incubated with VP-16 or ProVP-16 I and II. At indicated time points, cells were harvested and fixed in 4.5 ml ethanol (75%, -20°C) for at least 12 h (4°C). Cells were washed in PBS (pH 7.4) and resuspended in 250  $\mu$ l PBS containing RNase (0.3 mg/ml) and propidium iodide (50  $\mu$ g/ml) and incubated in the dark (30 min, RT). The DNA histograms defining distinct phases of the cell cycle were subsequently determined in duplicates by FACS analysis and average results were expressed as per cent.

**Toxicity studies in mice:** Stock solutions of 10 mM etoposide and proetoposide were prepared in 50:50 v/v cremophor:ethanol, and diluted to the final concentration in PBS (pH 7.4). A/J mice, 8 to 10 weeks of age were injected i.p. with 20 mg/kg or 60 mg/kg of either etoposide or proetoposide, or with solvent control (25% DMSO, 12,5% ethanol, 12,5% cremophor, 50% PBS), on day 1, 3, 5; 7, 9, and 11. VP-16 (60 mg/kg) was only injected on days 1,3 and 5. Body weights and survival were monitored over time.

**Anti-tumor effect in a T-cell leukemia xenograft model:** Primary tumors were induced by injection of  $5 \times 10^6$  MOVp 3 cells in 100  $\mu$ l PBS (pH 7.4) into the skin of the left lateral flank of each SCID mouse. Established primary tumors were palpable 55 days after injection. Mice (n=8) were treated by intraperitoneal injection of solvent (25% DMSO, 12,5% ethanol, 12,5% cremophor, 50% PBS), VP-16 (15mg/kg) and Pro VP-16 II (15 and 45 mg/kg) in a total volume of 200 $\mu$ l. Each mouse received a total of 7 injections on days 55,57,59,69,71,87,90 after tumor cell inoculation. Primary tumor size was determined over time by micro caliper measurements and volumes were calculated according to  $\frac{1}{2} \times \text{width}^2 \times \text{length}$ . Body weight was determined on a standard digital scale.

**Statistics:** The statistical significance of differential findings between experimental groups of animals was determined by two-tailed Student's t test. Findings were regarded as significant if two-tailed p values were  $<0.01$ .

**Chemistry of etoposide prodrug activation:** In order to establish that ProVP16-I and II are indeed prodrugs, we first determined their activation characteristics in vitro by HPLC (Fig. 1,2). Conversion of ProVP-16 I into ProVP-16 II and subsequent release of VP-16 follows a two step activation mechanism (Fig. 1). First, ProVP-16 I converts to ProVP-16 II with the elimination of 2,2-dihydroxypropane within 2 h with some degradation (about 10%) of the glycoside moiety under acid conditions (THF, 2N HCL) (Fig. 2A). Second, ProVP-16 II hydrolyses into VP-16 under basic pH conditions with the elimination of glycerine (Fig. 1,2B). In all experiments, conversion-time curves exhibited first order kinetics. Importantly, under physiological buffer conditions (PBS, pH 7.4, 37°C), ProVP-16 II is stable with a conversion rate of  $<5\%$  in 5 up to 18 h. ProVP-16 I is completely stable with no measurable conversion in PBS (pH 7.4, 37°C) and in contrast to ProVP-16 II, it is inert also under basic buffer conditions (pH  $\leq 10.0$ ). This unusual hydrolytic stability of ProVP-16 I is attributed to the hydrophobic nature of the entire southern region of this molecule and, to a lesser extent, also to steric hindrance from the two ortho methoxy groups surrounding the carbonate moiety.

The utility of the prodrugs according to the present invention is also evident from the fact that they are activated, i.e. hydrolysed in the presence of naturally occurring enzymes, but show stability in aqueous solutions over a wide range of pH-values.

Activation of ProPV-16 I and II occurs in the presence of serum with conversion half lifes of 750.8 min and 56.1 min, respectively. Porcine liver caboxyl esterase also mediated conversion of ProVP16 I and II into VP-16 with conversion half lifes of 14.2 min and 514.1 min, respectively. These findings clearly indicate enzymatic prodrug activation at pH 7.4 by carboxyl esterases (Table 1).

**Table 1.** Kinetic parameters for hydrolysis of etoposide prodrugs

compound	media (37°C $\pm$ 0,5)	PH ( $\pm$ 0,1)	k <sub>obs</sub> (10 <sup>-3</sup> -min <sup>-1</sup> ) <sup>a</sup>	t <sub>1/2</sub> (min)
Prodrug I	PBS buffer	5.0-10.0	no conversion	-



2		11.8	8.333 $\pm$ 0.50	83.16
	human serum	7.3	0.923 $\pm$ 0.046	750.8
	esterase <sup>b</sup>	7.3	48.89 $\pm$ 3.62	14.17
Prodrug II	PBS buffer	5.0-7.3	no conversion	-
3		8.0	3.201 $\pm$ 0.428	216.49
		8.8	7.142 $\pm$ 0.351	97.03
		10.5	100.01 $\pm$ 12.72	6.93
	human serum	7.3	12.36 $\pm$ 0.965	56.07
	esterase	7.3	1.348 $\pm$ 0.081	514.10

a) mean  $\pm$  SD

b) porcine liver carboxyl ester hydrolase

### Example 2

**Activity of ProVP-16 I and II against leukemia and cancer cell lines:** The cytotoxicity of these two proetoposides was tested against a panel of tumor cell lines using the XTT vital stain antiproliferation assay (Fig. 3A). For the majority of human tumor cell lines tested both proetoposides were substantially more active than the parent compound with IC<sub>50</sub> values 1000 fold lower with SW480 colon carcinoma, 100-1000 fold lower with HT-29 colon carcinoma, ADR5000 ovarian carcinoma and HL-60 pre B-cell leukemia, 10-100 fold lower with HeLa cervical carcinoma, A2780 ovarian carcinoma, K562 chronic myeloid leukemia, Jurkat T-cell leukemia, and SK-N-SH neuroblastoma, and 2-10 fold lower with Molt-3 T-cell leukemia, Reh and Nalm-6 pre B-cell leukemia, VCR100 T-lymphoblastic leukemia and CEM T-lymphoblastic leukemia cells. Only three cell lines, NXS2 murine neuroblastoma (Fig. 3A), CHO Chinese hamster ovary, and Hamms human colon carcinoma cells responded equally well to etoposide and proetoposides. Two cell lines, the doxorubicin-resistant ADR5000 and the vincristine-resistant VCR100, both with amplified MDR-1 expression, revealed a higher cytotoxic potential for both proetoposides than for the non-resistant parental cell lines A2780 and CEM.

The time course of cytotoxic action of Pro-VP16 I was followed by using Molt-3 T-cell leukemia cells (Fig. 3B). The results indicate a delayed onset of cytotoxic activity by ProVP-16 I, starting after 24 hr of incubation. At that time point, the cytotoxic effect of parental VP-16 was already almost completely established. The cytotoxic effect of ProVP16 I was completed 48-72 h after incubation (IC<sub>50</sub> 1.0x10<sup>-8</sup>) and exceeded that of VP-16 (IC<sub>50</sub> 6.5 x10<sup>-8</sup> M). Similar results were observed with ProVP-16 II (data not shown). These findings indicate a

slow release mechanism of cytotoxic activity from ProVP-16 I and II which is absolutely consistent with the prodrug concept.

### Example 3

**Effect of ProVP-16 I and II on multidrug resistant cells:** Based on the finding that ProVP-16 I and II are also more effective than VP-16 in natural MDR-expressing cell lines (Fig. 3), the question was addressed whether these prodrugs could overcome artificial MDR in vitro. For this purpose, the MDR-1 negative cell line Molt-3 was used to generate a resistant subclone MOV-3 (Fig. 4A). MDR-1 mRNA expression in MOV-3 cells was determined by RT-PCR (Fig. 4A) while increased MDR-1 protein expression on the cell surface was established by FACS-analysis using UIC2 monoclonal antibody. Extended gene expression analyses also looking at MRP, LRP, Topoisomerases I, II $\alpha$  and II $\beta$  as well as Bax and Bcl-2 (data not shown) reveal that the only difference accounting for drug resistance in MOV-3 cells in contrast to Molt-3 is expression of MDR-1. This was further confirmed by functional characterization of the subline revealed a 100-fold resistance against etoposide with IC<sub>50</sub> values of  $2 \times 10^{-6}$  M for MOV-3 and  $2 \times 10^{-8}$  M for Molt-3 cells (Fig. 4B). However, MOV-3 cells remained almost fully sensitive towards ProVP-16 I and II with no significant difference in etoposide mediated cytotoxicity between parental Molt-3 and drug resistant MOV-3 cells, with IC<sub>50</sub> values of  $2 \times 10^{-8}$  M for both prodrugs and cell lines. Similar results were obtained in Molt-3 cells following stable transfection with MDR-1 cDNA using pMDR-IRESpuro (Molt-3/MDR-1) that resulted in resistance against etoposide, doxorubicin, taxol and vinblastine (data not shown). Molt-3/MDR-1 cells also remained fully sensitive against ProVP-16 I and II. Controls stably transfected with empty vector (pIRESpuro) or a vector containing GFP revealed no resistance to any of the drugs tested.

Furthermore the type of resistance in the MOV-3 subline used was characterised by assessing the extent of cross resistance. MOV-3 cells displayed cross resistance against all MDR-1 type drugs (etoposide, doxorubicin, paclitaxel, vinblastine) in contrast to non-MDR-1 drugs (MTX, 5-FU, Genistein, Calicheamicin  $\theta$ ) (Fig. 4C). In summary, these findings clearly demonstrate that ProVP-16 I and II can overcome MDR-1 mediated multidrug resistance in vitro.

In a functional MDR-1 assay using JC-1 dye, an increased JC-1 efflux in MOV-3 cells could be demonstrated in contrast to Molt-3 controls as indicated by a decrease in the FL-1 signal in the resistant subline in contrast to Molt-3 parental cells (Fig. 4D). This decrease was abrogated by incubation with  $3 \times 10^{-4}$  M ProVP-16 I (Fig 4D) and inhibited

MDR-1 mediated efflux over a broad concentration range down to  $10 \times 10^{-6}$  M (data not shown). Interestingly, VP-16 used at equimolar concentrations was ineffective in modulating MDR-1 function as indicated by a JC-1 signal not different from PBS controls. These findings clearly indicate that the prodrug design directly decreases MDR-1 mediated substrate efflux.

In order to evaluate the cytotoxic mechanism mediated by ProVP-16 I and II in multidrug resistant MOVP-3 cells, the effect of these drugs on the cell cycle was analyzed at concentrations ranging from 10nM to 1  $\mu$ M and compared to Molt-3 parental cells. Typical results obtained with 0.5 $\mu$ M prodrugs are shown in Figure 5. Specifically, asynchronous MOVP-3 (Fig. 5 B) and Molt-3 (5 A) cells were incubated with VP-16, ProVP-16 I and II for 72 h. Periodically cells were subjected to cell cycle analysis at indicated time points. Results clearly indicate that both, Pro VP-16 I and II at 0.5 $\mu$ M (Fig. 5B) as well as for the entire concentration range (10nM-1  $\mu$ M) (data not shown) are very effective in inducing a steady increase in the pre-G1 peak in MOVP-3 cells after 24 h, characteristic of apoptosis. In contrast to the prodrugs, VP-16 was ineffective to induce apoptosis, consistent with elimination of VP-16, but not ProVP-16 I and II, by MDR-1. Induction of apoptosis was also demonstrated in Molt-3 parental cells over the entire concentration range (0.5  $\mu$ M, Fig. 5A). VP-16 was demonstrated to induce apoptosis in Molt-3 cells to a similar extent as ProVP-16 I and II. Overall, these results suggest that ProVP-16 I and II are no substrates for MDR-1.

#### Example 4

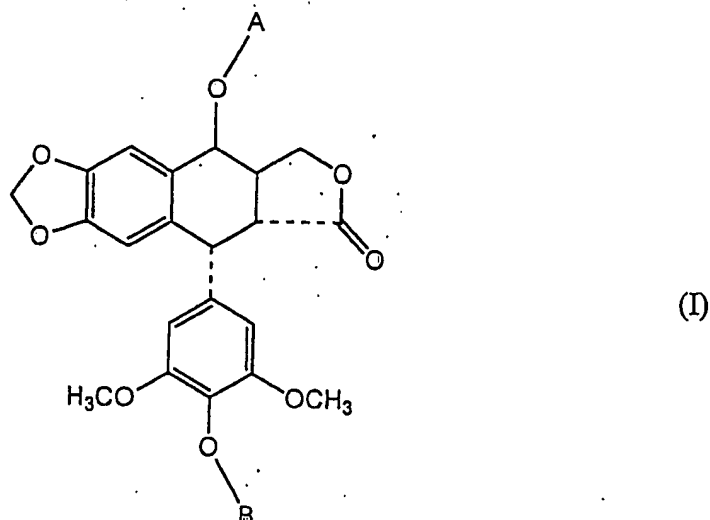
**In vivo toxicity and efficacy of ProVP-16 II in a multidrug resistant T-cell leukemia xenograft model:** Based on the in vitro findings that revealed no differences between ProVP-16 I and II, ProVP-16 II was selected for in vivo experiments because of higher water solubility. First, systemic toxicity of ProVP-16 II was determined in A/J mice (n=6) injected i.p. with VP-16 and ProVP-16 II (Fig. 6A). All mice receiving 20 mg/kg VP-16 survived with an average weight loss of 10%. In contrast, 5/6 mice treated with 60 mg/kg VP 16 showed a weight loss >20%. These findings sharply contrast with the results obtained with ProVP-16 II. In that case administration of 20 and 60 mg/kg ProVP-16 II was well tolerated with no death in either experimental group. Only mice receiving 60 mg/kg ProVP-16 II revealed a transient weight loss of <10 % in contrast to 20 mg/kg Pro VP-16 II, which maintained stable average body weights. Thus, the maximum tolerated dose defined by a decrease in body weight < 20% was established at 20 mg/kg for VP-16 and at 60 mg/kg for ProVP-16 II, consistent with a decrease of systemic toxicity of by the prodrug design by at least a factor 3.

Second, the anti tumor effect of ProVP-16 II was determined in a multidrug resistant xenograft model of T-cell leukemia and compared to VP-16. Established primary tumors were induced by s.c. injection of  $5 \times 10^6$  multidrug resistant MOVP-3 cells and tumor growth was followed over a time period of 105 days. Treatment was initiated 55 days after tumor cell inoculation by i.p. injection at an average tumor size of  $250 \text{ mm}^3$ . The dose levels for VP-16 (15 mg/kg) and ProVP-16 II (15 and 45 mg/kg) were selected based on results shown in Fig. 6A to further reduce systemic toxicity. Treatment with 45 mg/kg ProVP-16 II induced a regression of established primary tumors in 7/7 animals 10 days after initiation of treatment, which was stable for over 2 months (Fig. 6B). This treatment was well tolerated with a transient weight loss of only 6% (Fig. 6C). This finding was in contrast to that observed in mice treated with 15 mg/kg VP-16, which showed no anti-tumor response and revealed continuous primary tumor growth identical to control mice treated only with solvent. However, significant toxicity was observed in mice treated with 15 mg/kg VP-16 who exhibited a transient average weight loss of 20% (Fig. 6C). In fact, mice receiving 15 mg/kg ProVP-16 II showed no measurable weight loss (Fig. 6C) and presented with a dramatic reduction in primary tumor growth in contrast to mice treated with the equivalent amount of 15 mg/kg VP-16. In order to determine whether MDR-1 expression remained stable over the course of the experiment, RNA was isolated from tumor explants at day 105 and all tumors investigated revealed an MDR-1 signal by RT-PCR (Fig. 6D).

The features of the present invention disclosed in the specification, the claims and/or in the accompanying drawings may, both separately and in any combination thereof, be material for realising the invention in various forms thereof.

Claims

1. A podophyllotoxin represented by formula I

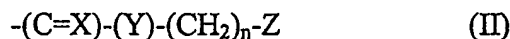


wherein A is H or is selected from the group comprising carbohydrates, polyols,  $-(\text{CH}_2-\underset{\text{OH}}{\text{CH}}-)_p$  H,  $\text{H}-(\text{CH}_2-\underset{\text{OH}}{\text{CH}}-)_p$ , ethylene glycol, propylene glycol, glycerol, penta-

erythritol, polyethyleneglycol and compounds, as represented by formula III below,

wherein p is an integer from 2 to 100, and

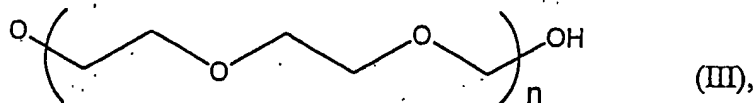
wherein B is represented by formula II



wherein X is selected from the group comprising O, S and  $\text{NR}''$ , Y is selected from the group comprising O, S, and  $\text{NR}''$ , wherein  $\text{R}''$  = alkyl, aryl or H,

n is an integer of from 0 to 6; and

Z is a polyhydroxyalkyl group selected from the group comprising ethylene glycol, propylene glycol, glycerol, pentaerythritol, polyethyleneglycol and compounds represented by formula III



or Z is a polyhydroxyalkyl group, as defined above, which additionally has a dioxolane group attached,

or Z is a dioxolane group,

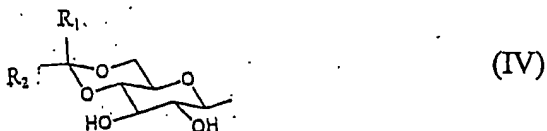
or Z is selected from the group comprising targetting moieties for mammalian receptors, antibodies, steroids, transferrin, proteins and peptides having tumor cell associated receptor finding function,

2. A podophyllotoxin according to claim 1, characterized in that the dioxolane group is selected from the group comprising 2,2-dialkyl-1,3-dioxolane, wherein each alkyl at the 2-position is independently selected from the group comprising unsubstituted and substituted methyl, ethyl, propyl, butyl, pentyl and hexyl.

3. A podophyllotoxin according to any of claims 1-2, characterized in that Z is a targeting moiety for a mammalian surface membrane receptor or for a mammalian nuclear receptor, or Z is an antibody, steroid, transferrin, protein or peptide having tumor cell associated receptor binding function.

4. A podophyllotoxin according to claim 3, characterized in that Z is selected from the group comprising steroids, growth factor receptor inhibiting proteins, peptides and non-peptide mimetics.

5. A podophyllotoxin according to any of the foregoing claims, characterized in that A is selected from the group comprising compounds as represented by formula IV

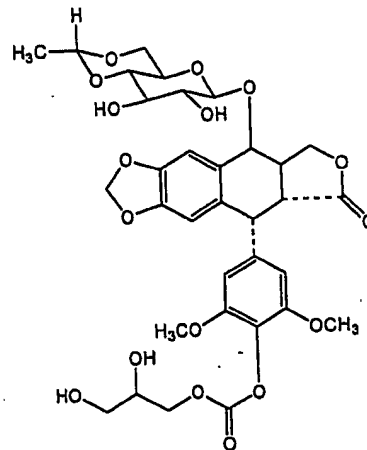
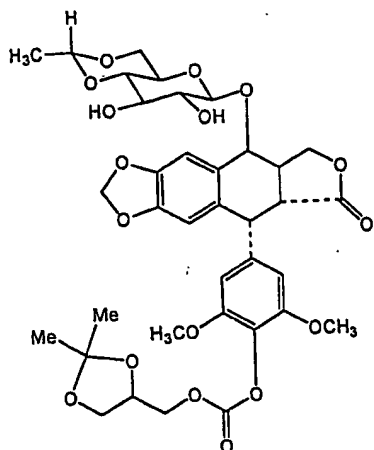


wherein R<sub>1</sub> and R<sub>2</sub> are each C<sub>1</sub>-C<sub>10</sub> alkyl; or R<sub>1</sub> and R<sub>2</sub> and the carbon to which they are attached represent C<sub>5</sub>-C<sub>6</sub> cycloalkyl; or R<sub>1</sub> is H and R<sub>2</sub> is selected from the group comprising C<sub>1-10</sub> alkyl, C<sub>2-10</sub> alkenyl, C<sub>3-5</sub> cycloalkyl, furyl, thienyl, C<sub>6-10</sub> aryl, and C<sub>7-14</sub> aralkyl.

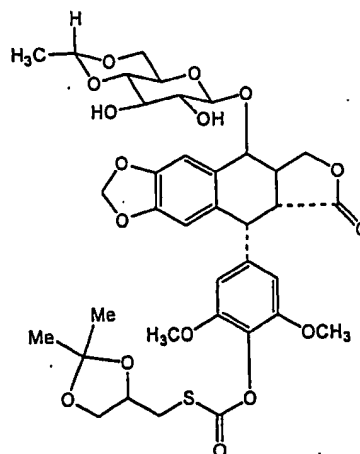
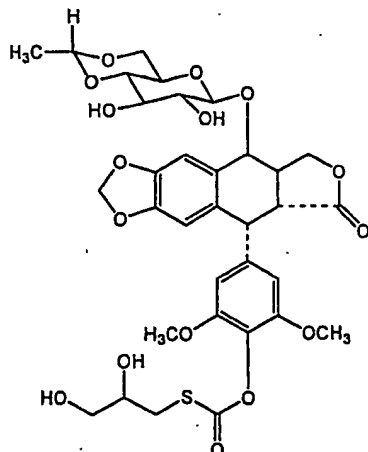
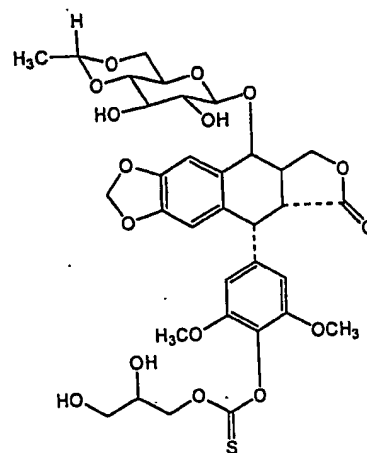
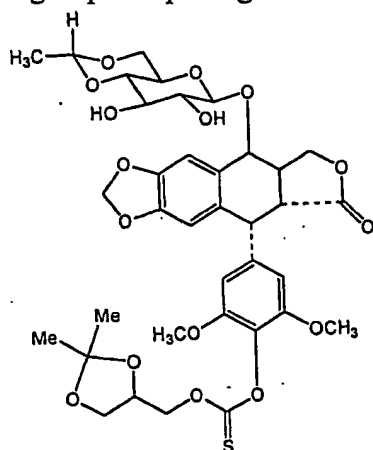
6. A podophyllotoxin in according to claim 5, wherein R<sub>1</sub> is H and R<sub>2</sub> is methyl or thienyl.

7. A podophyllotoxin according to any of the foregoing claims characterized in that X is O and Y is O.

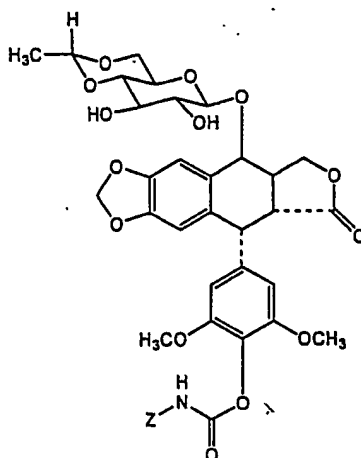
8. A podophyllotoxin according to any of claims 1-6 characterized in that X is O and Y is S.
9. A podophyllotoxin according to any of claims 1-6, characterized in that X is O and Y is NH.
10. A podophyllotoxin according to any of claims 1-7 characterized in that it is selected from the group comprising



11. A podophyllotoxin according to any of claims 1-6 and 8 characterized in that it is selected from the group comprising

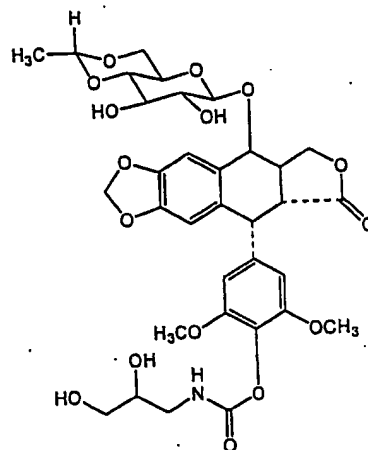
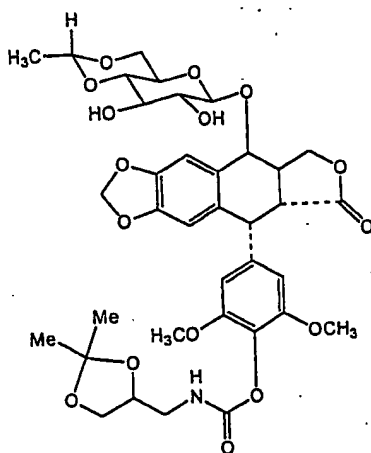


12. A podophyllotoxin according to any of claims 1-6 and 9 characterized in that it is selected from the group comprising

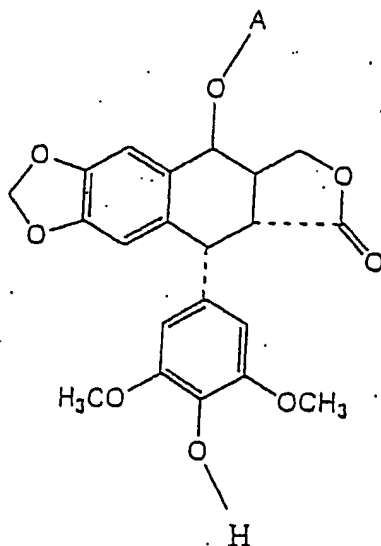


wherein Z is defined as in claim 1.

13. A podophyllotoxin according to claim 12, characterized in that it is selected from the group comprising



14. A method of preparing a podophyllotoxin according to any of claims 1-13, characterized in that a compound as represented by formula V



(V)



A being as defined in any of the foregoing claims,

is reacted with a haloformate  $W-(C=X)-(Y)-(CH_2)_n-Z$ , wherein X, Y, Z and n are as defined in the foregoing claims, and W being Cl, F, Br or I,

or characterized in that a compound as represented by formula V is reacted with phosgene or trichloromethylchloroformate, to yield a 4'-phenol chloroformate intermediate,

said 4'-phenol chloroformate intermediate then being reacted with an alcohol or thiol of the formula ZYH, to yield the corresponding carbonate or thiocarbonate,  $Y=O$  or S, and Z being as defined in the foregoing claims, or

said 4'-phenol chloroformate intermediate then being reacted with an amine of the formula  $HNR''Z$  to yield the corresponding carbamate,  $R''$  and Z being as defined in the foregoing claims.

15. A method according to claim 14, characterized in that the compound as represented by formula V is reacted with a compound selected from the group comprising p-nitrophenyl soketalcarbonate, soketalchloroformate, and PEG-chloroformate.

16. A method of preparing a podophyllotoxin according to any of claims 1-13, characterized in that the product resulting from any of claims 14-15 is hydrolyzed.

17. Pharmaceutical composition comprising a podophyllotoxin according to any of claims 1-13 or of a podophyllotoxin prepared according to any of claims 14-16, and a pharmaceutically acceptable carrier.

18. Use of a podophyllotoxin according to any of claims 1-13 or of a podophyllotoxin prepared according to any of claims 14-16 or of a pharmaceutical composition according to claim 17 for the manufacture of a medicament for the treatment of cell proliferative disorders.

19. Use according to claim 18, characterized in that the cell proliferative disorder is in a child, an adolescent or an adult.

Fig. 1

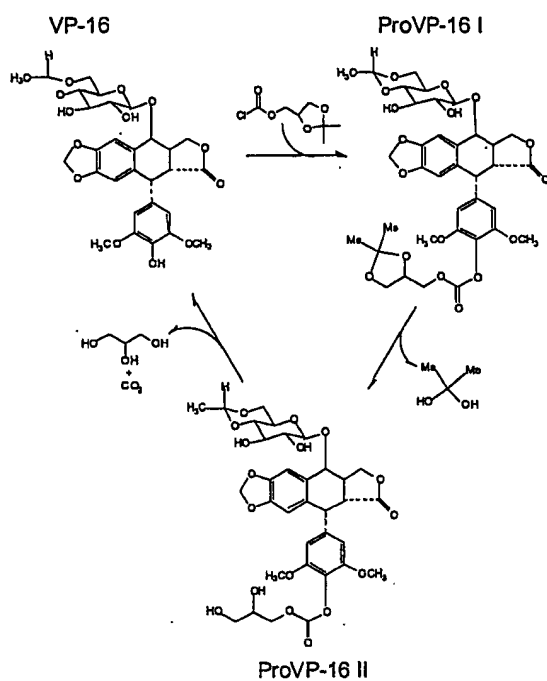


Fig. 2

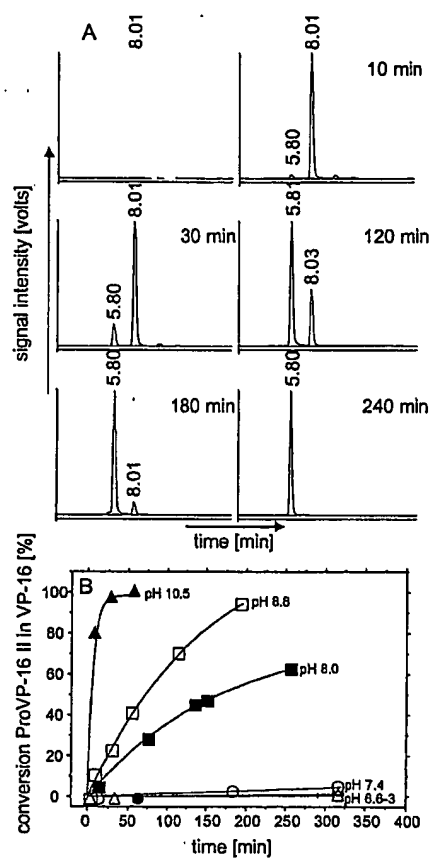


Fig. 3

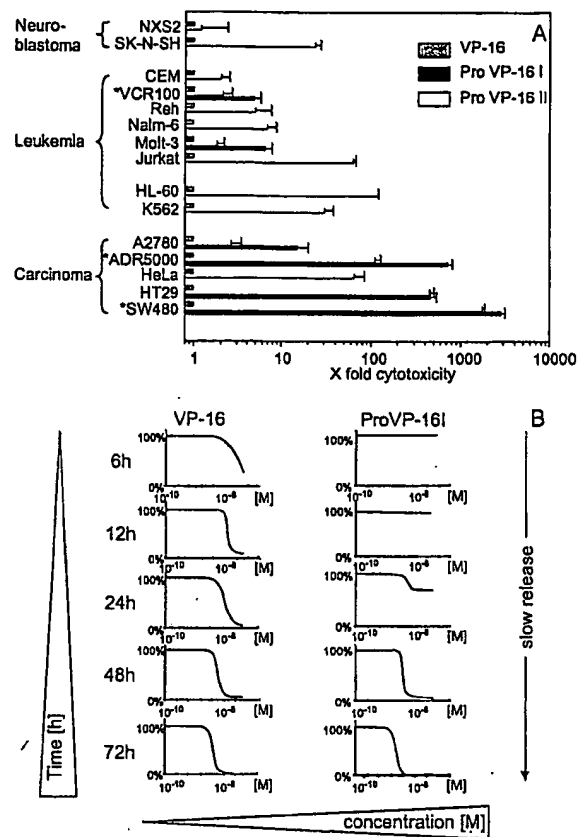


Fig. 4

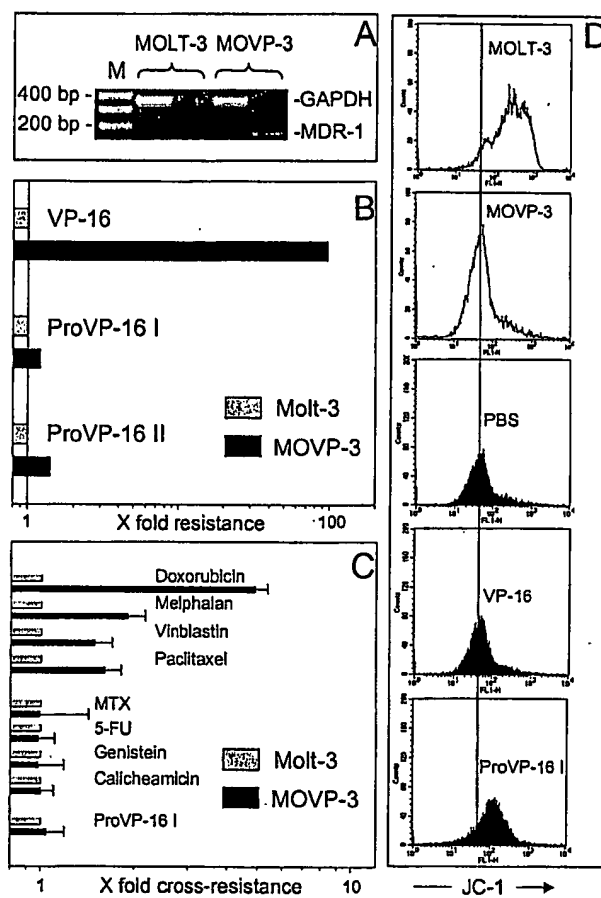


Fig. 5

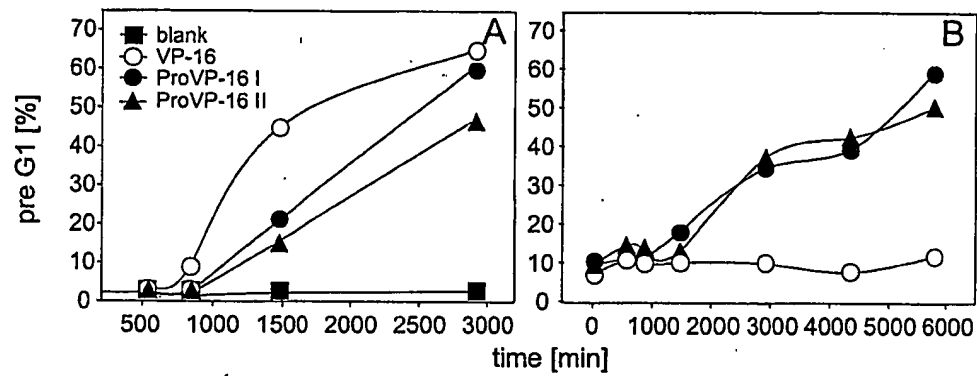
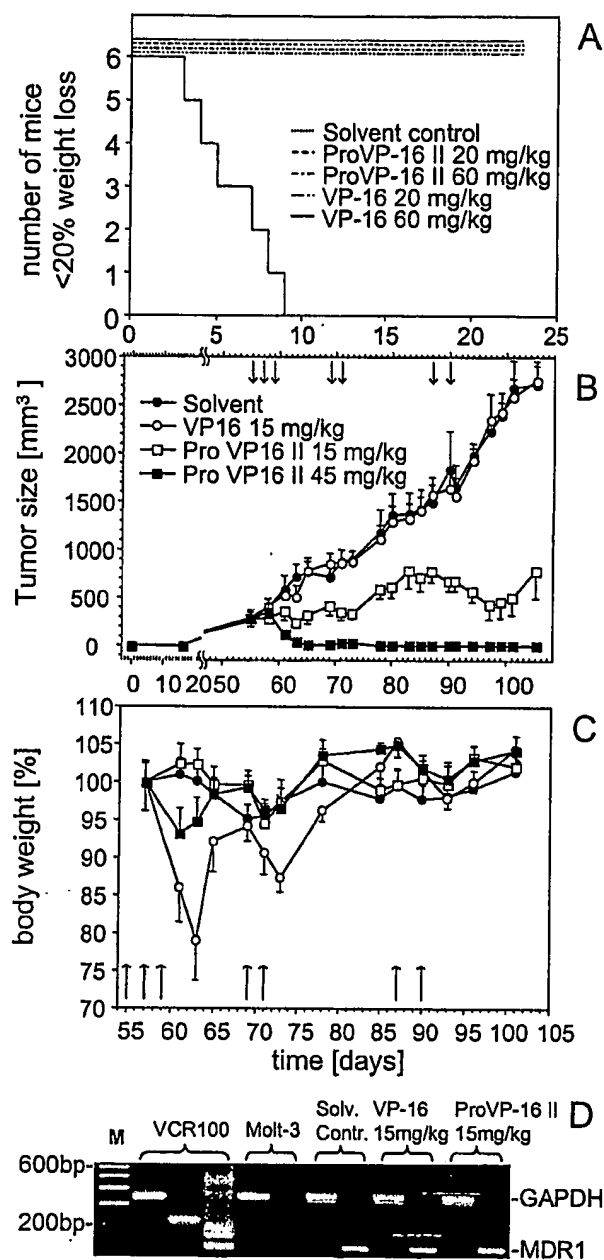


Fig. 6



## A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C07D493/04 A61K31/34 A61P35/00

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C07D A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

CHEM ABS Data, BEILSTEIN Data

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	A. NIETHAMMER ET AL.: "Synthesis and Preclinical Characterization of a Paclitaxel Prodrug with Improved Antitumor Activity and Water Solubility" BIOCONJUGATE CHEM., vol. 12, no. 3, 27 April 2001 (2001-04-27), pages 414-420, XP002232620 figure 1 -----	1-19
A	US 5 681 567 A (A. J. MARTINEZ, R. B. GREENWALD) 28 October 1997 (1997-10-28) claims 1,26 -----	1-19

## INTERNATIONAL SEARCH REPORT

In — onal Application No

PCT/EP 02/13669

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